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Physiological Zoölogy

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SOME GROWTH-REGULATING FACTORS IN TUBULARIA

(Fifteen figures)

FLORENCE PEEBLES[1]
California Christian College, Los Angeles

THE series of changes which result in what is termed the "growth" of an organism are directed and controlled through the interaction of internal and external conditions. Increase in size, which is one manifestation of growth, may depend largely upon food supply; so, too, the process of reproduction is directly related to this external condition. The development of the egg into tissues and organs, although dependent upon internal organization, is greatly modified by external forces such as pressure, light, temperature, and chemicals; but growth and differentiation are also manifestations of an inherent capacity of living matter, and the "growth impulse" as Mendel (Greene, 1928) once described it, however shaped and modified it may be by external conditions and exchanges of material, continues its course as a controlling factor in the organism from its earliest beginning.

Such an organism as the tubularian hydroid is well adapted to the experimental study of these processes because it possesses the growth impulse to a marked degree and because this impulse manifests it-

[1] The investigations were begun while occupying the American Woman's Table in the Zoölogical Station at Naples, in July and August, 1927, and were continued at the Scripps Institution of Oceanography, La Jolla, California, from July to September, 1928. I am glad to acknowledge here my appreciation of these opportunities to carry on the work.

self readily under experimental conditions. That it is a favorable form for study is evident when one reviews the extended literature describing the investigations already made—from the early publications of Loeb, Driesch, and Morgan, to the more recent work of Child, Lund, and Hyman. The early experimental studies dealt principally with the phenomena of regeneration per se, but more recently attention has been given to this process only in so far as it leads to the regulation of body form and the completion of the unit system. As such it resolves itself into a part of the general problem of growth and organization. Driesch considered regeneration from the standpoint of its *Reparationsfähigkeit,* while Child and Hyman approach it from the viewpoint of the axial gradient and of the regulative processes which control the unity of a system dominated by an apical region of intense metabolic activity.

It is the purpose of the investigations described here to show, if possible, that the ability of the organism to complete itself may be measured in terms of its energy capacity rather than in units of volume, and that the axial gradient exhibited in isolated stems is largely the expression of the metabolic activity of the part at the time of isolation and during the period of the experiment.

EXPERIMENTS

I. THE EFFECT OF THE REMOVAL OF ONE END OF THE STEM AFTER THE WOUND HAS CLOSED

a) *Removal of the distal end.*—In the first series of experiments stems of 4–6 cm. were cut from fresh vigorous colonies as soon as they were brought into the laboratory. The hydranths, together with 1–2 mm. of the distal end, were removed (Fig. 1, *A*, first operation). The wounds were given time to close; then a short piece, 4–6 mm. in length (*a–b*), was cut from the distal end (*B*). At the same time the hydranth was removed from other stems (*C* and *D*) at corresponding levels, to serve as controls for *B*. There were thus four distal cut surfaces for comparison: *a* and *a'*, *c* and *c'*. The rate of emergence of the hydranths and their size were compared. In each case the second cut was made before there was any indication of the primordium. The period between the first and second operations varied from 2 to 13 hours. The small distal piece (*a–b*) was much longer

than the primordium of a full-sized hydranth; so that it was always possible to determine whether or not the hydranth that formed from it was already laid down at the time of the second operation. If it was not already determined, the new hydranth was smaller than that at a' and slower in emerging from the perisarc.

Child (1914) has shown, in *Planaria dorotocephala*, that the fate of a small piece taken from the region just back of the pharynx is not determined at the time it is isolated from the body, although the processes which determine head formation in such pieces begin immediately. Six to eight hours after isolation no other sort of structure is produced, and head formation in such pieces cannot be prevented. The case is somewhat similar in *Tubularia*, although a new hydranth forms at the cut end (Fig. 1, *a*) in 100 per cent of the pieces; the size of the structure and the rate of its appearance indicate whether or not the piece *a–b* is acting as a part of the entire system (*a–d*). If *a–b* acts as an independent small piece, the hydranth that emerges from it should appear at about the same time as the one at *c*, and it should be of smaller size. Also, if the formation of a new hydranth at the distal end of the stem inhibits the process of hydranth-building at the proximal end, the removal of the piece *a–b* should remove this inhibiting influence, and the new basal hydranth at *d* should then appear sooner.

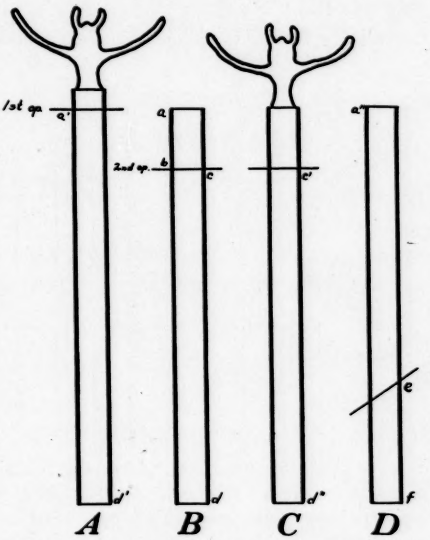

FIG. 1.—Stems from which a small distal piece has been removed after reconstitution has begun. *A* (first operation) shows removal of the original hydranth in the experimental stem; *C*, the removal of the hydranth in the control stem. *B* shows the level of the second operation; *D*, the control for *B*. In *D* the cutting-off of the proximal piece (*e–f*) is also shown.

In Figure 2 ten stems were cut as just described. The period between the first and second operations was 2 hours; the average length of the small distal piece (*a–b*) was 4.5 mm.; of the longer proximal piece (*c–d*), 35 mm. A comparison of the results on the first and second days after the operations (Fig. 2) shows that all of the small pieces (*a–b*) formed medium-sized or small hydranths, thus proving that they behaved as separate units. At the end of the first day, seven of the short pieces showed primordia and two hydranths were fully formed. Primordia had formed also on the pieces used as

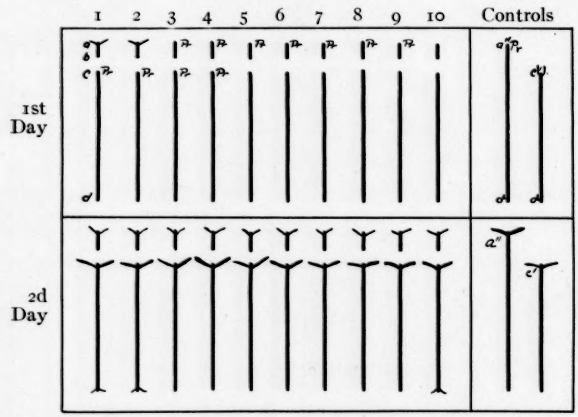

FIG. 2.—Diagram of stems from which short proximal pieces were cut off 2 hours after the stems were isolated and the original hydranth removed. The series shows growth of new hydranths 24 and 48 hours after the second operation.

controls. The development at the distal end (*c*) of the long piece was slightly behind that of the control. The proximal ends showed no development at the end of the second day, but 100 per cent of the large and small pieces produced complete oral hydranths. Those on the small pieces were all of medium size; those on the long pieces were equal in size to those on the controls. The proximal ends of the short pieces showed no formation of either hydranths or stolons, and only two of the long pieces developed basal hydranths. It will be noted that these two stems are the ones that developed the complete hydranths before any of the others, thus indicating a more vigorous condition at the time of the operation.

In Figure 3 another series of ten pieces is represented. Here the second operation followed the first after a period of 7 hours instead of 2. The average length of the short pieces was 5 mm.; of the long piece, 45 mm. There is strong evidence here that the small piece is

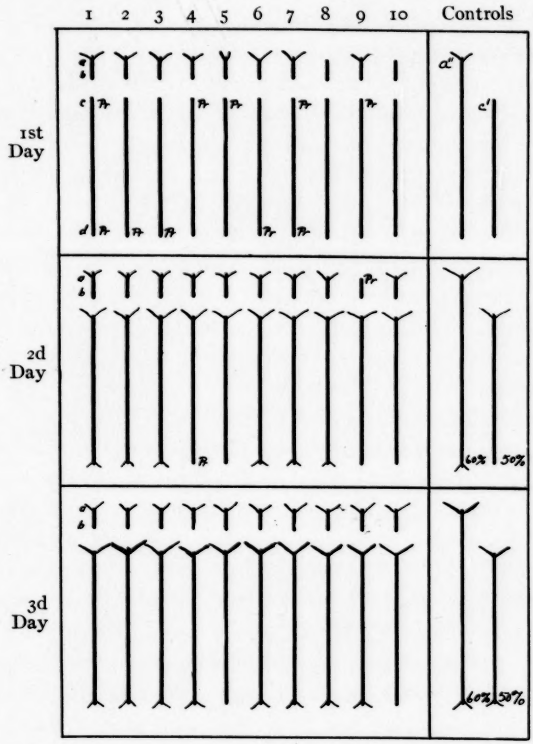

FIG. 3.—Same as Figure 2. The period between first and second operations was 7 hours.

acting as a part of the original stem; although the size is reduced, the hydranths in 80 per cent of the small pieces were out or nearly out before the hydranth at *c*. The activity at the end (*a*) of the small piece evidently produced an accelerating effect on *c*, as 50 per cent of the hydranths at *c* were laid down before the primordium ap-

peared at the corresponding level (*c'*) of the control. There is also some evidence that removal of the piece *a–b* hastens the formation of the basal hydranth at the end *d*. Here 50 per cent of the pieces formed primordia before they appeared on the basal ends of either control. The size of the hydranth at the distal end of the piece *a–b* in no case equaled that of the distal hydranth on the piece *c–d*. Basal hydranths never formed on the short pieces, but a larger percentage of basals appeared on the operated pieces (*c–d*) than showed on the controls. This is indicated on the second and third days (Fig. 3).

In a third series, shown in Figure 4, the small piece was isolated 13 hours after the removal of the hydranth. All but one of the small pieces here behaved as part of the whole stem, forming large hydranths at *a* before they appeared at *c*. A tremendous increase in rate of appearance and in number of basal hydranths is evident, as 100 per cent formed on the experimental stems and only 40 per cent on the controls. It is evident from comparing these tables that hydranth formation is determined several hours before the primordium appears, and it is so far advanced by the thirteenth hour that the small piece builds as large a hydranth as it would were it still a part of the whole stem. Secondly, the formation of a large hydranth at *a* in no way alters the size of the hydranth in *c* when the period between the first and second operations is increased. There is definite evidence of delay at the distal end of the long piece, caused, no doubt, by basal inhibition, since by the thirteenth hour the hydranth at *a* has begun to develop. Thirdly, it is possible as late as the thirteenth hour after the removal of the original hydranth for the small piece (*a–b*) to readjust itself; and although the primordium of the hydranth has been begun, the piece is still capable of behaving as a short separate piece with a small hydranth at each end. This happens very seldom but is shown in the first piece in this series (Fig. 4, 1). These experiments were repeated ten times, making one hundred stems in all. From this number an average of 95 per cent of the short pieces behaved as a part of the original stem, and between 90 and 95 per cent of the basal ends produced hydranths.

b) *Removal of the proximal end.*—In a second series of experiments the basal end of the stem was removed, as shown in Figure 1, *D*.

In order that the oral and aboral ends of the short pieces (*e–f*) should not be confused, the cut at *e* was made slightly oblique. This does not delay or greatly modify development, although it does influence

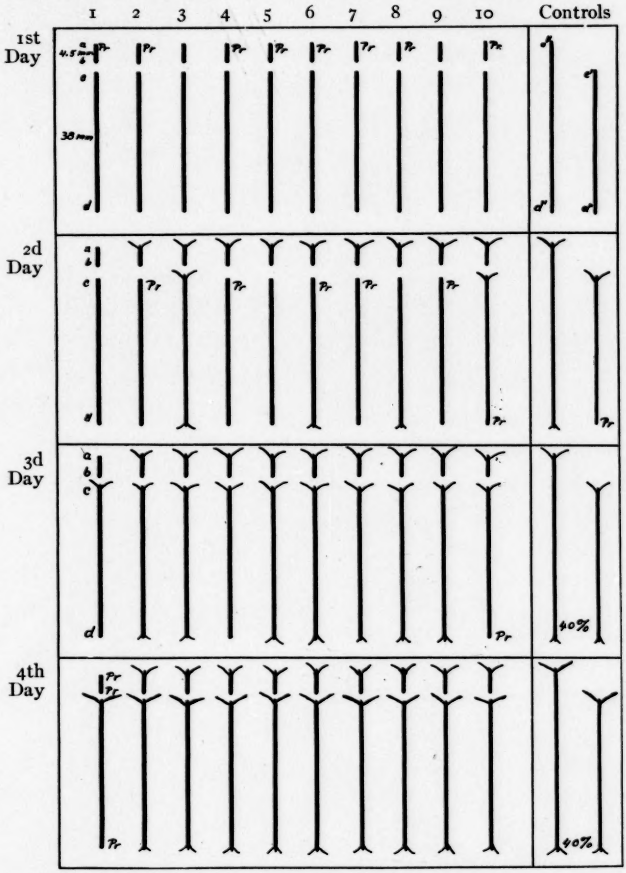

FIG. 4.—Same as Figure 2. The period between operations was 13 hours

the direction of growth of the stalk of the new hydranth (Peebles, 1900). Since the basal hydranth always develops more slowly than the apical, it was considered unnecessary to test the effect of a very short period between the first and second operations. Figure 5 shows

the effect of removal of the basal end 5 hours after isolation of the entire stem. The average length of the pieces was about the same as that of the first experiment. Comparing Figures 3 and 5 twenty-four hours after the removal of the short pieces, it is evident that the metabolic activity of *a–b* is much greater than that of *e–f;* also, that the polarity of the small piece *e–f* at the basal end of the stem is less marked than that of the distal piece (*a–b*). In the third place the small piece *e–f* frequently forms a hydranth at both ends, while this seldom takes place in a short piece from the distal end of the stem.

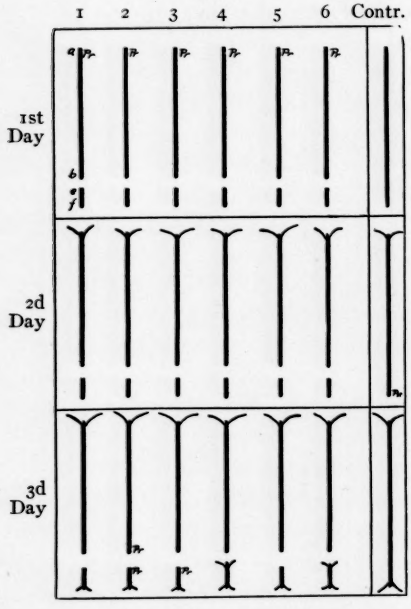

FIG. 5.—Diagram of reconstruction of stems from which the proximal end (*e–f*) has been cut off 5 hours after removal of the hydranth.

In Figure 6, where the period between the first and second operations was 16 hours, it will be seen that a larger percentage of the small pieces (*e–f*) formed distal hydranths on the second day. It must be remembered, however, that 1 day after the second operation in this series means that the basal end of the stem had been cut from the colony 40 hours before, while in the series given in Figure 5 the time was only a little more than 1 day. It is evident that the small pieces in Figure 6, by longer contact with the main stem, have retained their original polarity and behave as parts of the whole stem, while those removed after a shorter period of contact with the stem are able to proceed with the aboral hydranth because of the removal of the inhibiting influence of the apical end. These results indicate that the longer the basal piece remains in connection with the

remainder of the stem while it is forming an apical hydranth the greater the power of the small piece to produce new apical structures, but fewer basal ones are formed. These results are probably brought about in the first case by removal of apical dominance and in the second place by the longer action of this inhibition. The hydranths formed on the small piece (*e–f*) are never as large as those at the apical end of the longer piece. Comparing their size with those on the apical pieces, *a–b* in the first experiment, one finds that they are extremely small. The basal pieces, however, show a greater tendency to form a hydranth at both ends than do the apical pieces. This may be interpreted as equal amount of energy divided between the two ends, and this division may be brought about by the antagonistic influence of polarity and wound stimulation at *f*, while the increase in the number of basal hydranths may be due to the removal of apical dominance before development has begun so that the wound stimulus at *f* results in hydranth formation.

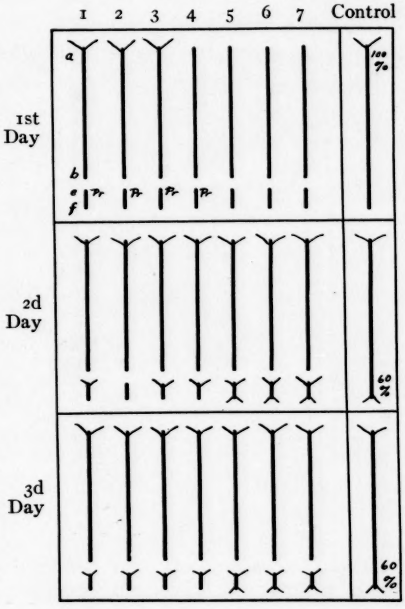

FIG. 6.—Same as Figure 5. The period between operations is 16 hours.

II. THE BEHAVIOR OF VARIOUS REGIONS OF THE STEM UNDER SIMILAR CONDITIONS OF WOUND STIMULATION

a) *Division of entire stem.*—According to the gradient theory of Child (1907–29[1]), as shown by his own work and that of Hyman

[1] The literature on this subject has covered such a long period and is so extended that reference here will be made to only a few of the papers bearing upon special experiments. A comprehensive list of the literature is given by Child (1929).

(1920, 1926), Lund (1921), and others, the region of the stem immediately below the hydranth is the part where metabolic activity is at its highest point. This has been demonstrated again and again, in the rate of development of hydranths, in susceptibility to chemicals, and in electrical behavior. Also, it is definitely established that the rate of oral hydranth formation is greater in distal than in proximal pieces. All experiments prove that the cut surface is a definite stimulus which calls forth a response in the form of a complete oral hydranth followed by an aboral one.

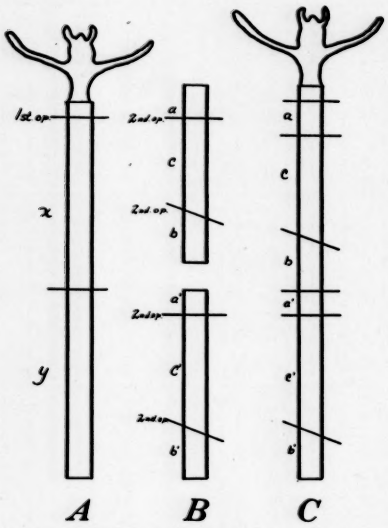

FIG. 7.—Stems cut into long and short pieces. In *A* the original hydranth is removed; in *B* the stem is cut into six pieces (*a*, *b*, *c*, *a'*, *b'*, and *c'*); in *C* the stem is divided at the same time the hydranth is cut off; *x* and *y* indicate the division of the stem into halves before each half is divided into three pieces.

In order to observe the reaction of cut surfaces at various levels along the stem, the following experiment was made. From long vigorous stalks of the same colony the hydranth and the basal portion were removed (Fig. 7, *A*). The ends of such pieces were allowed to close in, and after 6–15 hours a second operation was performed, dividing the stem into long and short pieces, as shown in Figure 7, *B*, *a*, *b*, *c*, and *a'*, *b'*, *c'*. The piece *a* thus has a start of several hours and is presumably beginning to form a new hydranth. The small piece *b'* has been exposed at its proximal end to the stimulating influence of the other cut surface; but, as we know from the observations of many experimenters, the hydranth formation here is delayed and there may not be any further change than closure of the wound. At the time of the second operation control pieces were cut, so that fresh stems were divided into exactly the same number of pieces;

but in this case the basal end and the hydranth were removed at the time the stem was divided into the sixth regions (Fig. 7, *C*, *a*, *b*, *c*, *a'*, *b'*, *c'*). In this way all the ends in the controls were exposed to wound stimulation simultaneously. The later development of all the pieces was compared during a period of 3 or 4 days, with the behavior of the pieces of the stem in Figure 7, *B*. Table I gives the length of the pieces, and the percentage of new structures developed at the various levels. The periods between the first and second operation varied from 6 to 15 hours.

TABLE I

PERCENTAGE OF HYDRANTHS AND STOLONS ON STEMS CUT INTO LONG AND SHORT PIECES AFTER FIRST ALLOWING THE DISTAL AND PROXIMAL ENDS OF THE STEM TO CLOSE

No. of Stems	Average Length of Piece (Mm.)	Level of Piece	Percentage of Apical Hydranths				Percentage of Basal Hydranths				Percentage of Basal Stolons			
			6 Hrs.	13 Hrs.	15 Hrs.	Control	6 Hrs.	13 Hrs.	15 Hrs.	Control	6 Hrs.	13 Hrs.	15 Hrs.	Control
10.....	4.5	*a*	100	100	100	100	0	0	0	0	0	10	10	0
10.....	9.0	*c*	100	100	100	100	30	100	70	20	0	0	0	0
10.....	4.0	*b*	100	100	100	100	40	30	50	20	20	0	20	20
10.....	4.5	*a'*	100	100	100	100	60	30	10	20	20	10	0	20
10.....	11.0	*c'*	100	100	100	100	50	90	60	60	0	0	10	0
10.....	10.0	*b'*	100	100	100	100	50	90	70	20	20	0	0	20

Comparison of these records shows, very definitely, that basal hydranths failed to form on short pieces from the extreme distal end of the stem. The growth impulse here is directed away from the base. Such pieces, although they come from the most vigorous region of the stem, do not as a rule develop stolons. In several hundred stems stolons formed in this region in only one or two sets, and that was at a season when the colonies were breeding and were in an unusually vigorous condition. In the majority of cases where stolons developed at all they were found on pieces from the middle or basal regions and were on short rather than on long pieces. There was definite variation in the size of the hydranths at the different levels. Oral hydranths, if on *a*, were always the largest, provided the period between the first and second operations was longer than 12 hours. If the period was short, the oral hydranth on *c* was the largest. On *b* and *a'* they were smaller than those on *a* and *c*, but were about equal

to each other. Those on c' were large, and on b' much smaller than c and c'. Frequently two hydranths of equal size appeared on both ends of b and a'; less frequently on b'. There was little variation in the size at the various levels in the control except that the oral hydranth on c was always larger than that on a. In the basal piece (b') the majority of the proximal ends formed no hydranths in the control, but in the operated stems from 50 to 90 per cent produced hydranths at the basal end on the second and third days. This was true to a less extent on the pieces b and a'. In the control the majority of these pieces failed to form basal hydranths by the third day, while on the operated stems 40 to 60 per cent developed basal hydranths on the second day. This indicates a stimulating of metabolic processes through the closure of the wounds at each end and the activation of formative processes at the oral extremity. In contrasting the total number of apical hydranths on the experimental stems and the control, it will be seen that every piece developed a hydranth; but the total number of basal hydranths on the experimental stems was double that of the control, while the number of stolons was no greater than on the control.

It is very definitely demonstrated by comparing the behavior of pieces from the various levels of the stem that there is much individual variation in stems, and this must be taken into consideration in estimating both rate and percentage of development. The strong vigorous stem may develop new structures several hours ahead of a weak inactive one; and, as has been mentioned elsewhere, the rate of formation and the character of the structures at a given temperature vary with the seasons. In July and August *Tubularia mesembryanthemum*, at Naples, and *T. crocea*, at La Jolla, both show signs of depression; but toward the end of August and during the first week in September, when the reproductive period sets in, *T. crocea* becomes tremendously vigorous, and at this time a much larger percentage of stolons is obtained. This supports Child's statement (1907*a*) that hydranths develop when the conditions are not sufficiently favorable for stolon formation.

b) *Division of half-stems.*—The experiment just described was slightly modified by first dividing the stem into halves (Fig. 7, *A*, *x* and *y*) and then allowing the cut ends to close in and development

to proceed for a period of 15 hours before the second operation (Fig. 7, *B*). Each half was then cut into three pieces as before. The modification consisted in exposing the basal end of *b* and the distal end of *a'* to the same conditions of wound stimulation. The larger pieces (*c* and *c'*) were not subjected to any external influence, yet *c'* in this case was nearer the region of activity than before. Table II shows the results of a series of half-stems.

If the controls in Tables I and II are compared, it will be seen that the stem cut in half did not develop as large a percentage of

TABLE II

PERCENTAGE OF HYDRANTHS AND STOLONS ON PIECES OF STEMS CUT, FIRST IN HALF, AND THEN, AFTER CLOSURE OF THE ENDS, DIVIDED INTO THREE PIECES

LEVEL OF PIECES	NO. OF PIECES		AVERAGE LENGTH (Mm.)		PERCENTAGE OF APICAL HYDRANTHS		PERCENTAGE OF BASAL HYDRANTHS		PERCENTAGE OF BASAL STOLONS	
	Exp.	Control	Exp.	Control	Exp.	Control	Exp.	Control	Exp.	Control
a	10	10	7	35	100	100	0	.....	60	0
c	10	.....	18	.....	100	.....	80	.....	20	.....
b	10	.....	10	.....	100	.....	30	80	30	.....
a'	10	10	7	40	100	100	0	.....	70	0
c'	10	.....	17	.....	100	.....	70	.....	0	.....
b'	10	.....	8	.....	100	.....	60	40	10	.....

basal structures as those cut into many pieces. In other words, stems divided into shorter pieces produce more basal structures at a given level. This is, no doubt, due to the changes brought about by wound stimulation.

III. REPEATED REGENERATION AFTER LIGATURING IN THE MIDDLE OF THE STEM

Assuming that the region of the stem distal to the middle possesses greater hydranth potency and a higher metabolic rate than the proximal half, we should expect that larger hydranths would arise from the distal half, that they would develop more rapidly, and that, if removed as soon as formed, those on the distal half would be larger than those on the basal ends. This does not always happen, as the following experiment will show.

After removal of the hydranth and 1–2 mm. of the stem, as in the first experiment (Fig. 1, *A*), a silk thread was tied around the center,

thus cutting off all communication between the coenosarc of the distal and proximal halves of the piece. This experiment was made first by Morgan (1902) and later by Loeb (1904) and Morgan and Stevens (1904). Their results all go to prove that cutting off the influence of the distal end tends to hasten the development of the proximal end, so that the time period between the formation of the two hydranths is greatly reduced.

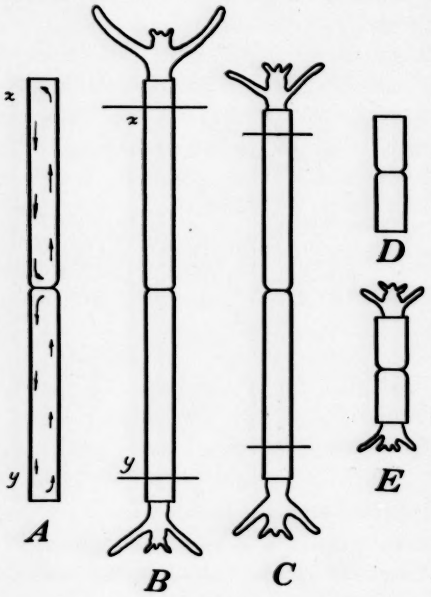

FIG. 8.—Stems ligatured in the middle, dividing the stem into a distal half (x) and a proximal half (y). In A arrows indicate the circulation of granules. B and C, the removal of the new hydranths as they appear. D and E, the approach of the levels of the cuts to the ligature with the resulting uniform size of the hydranths.

Shortly after the ligature is made, the wounds at each end close in, and the granules can be seen circulating in each half as indicated by the arrows in Figure 8, A. Wound stimulation exerts its influence at x and y only, as there is no opening at the middle of the stem. As soon as the hydranths emerged on either end, they were immediately cut off (Fig. 8, B and C); and this process was repeated until the halves were reduced to a length of less than 1 mm., when no further regeneration took place.

In Figure 9 the history of ten ligatured stems for a period of 12 days is given. It will be noted that at first the apical hydranth usually precedes the basal one in formation, and it is always larger the first time it is formed; but as each hydranth is removed, the succeeding ones appear at about the same time, and the third and fourth hydranths at each end appear almost synchronously and are

generally of equal size. The actual volume of the halves differs very little, but it becomes more nearly the same as the level of the cut

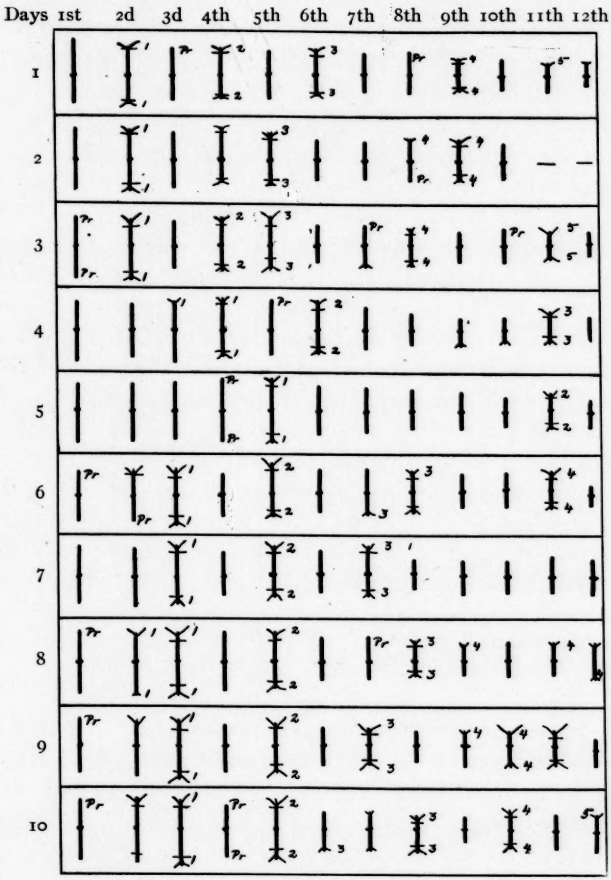

FIG. 9.—Repeated hydranth formation on stems ligatured in the middle. The figures in the vertical column represent the number of the pieces; in the horizontal column, the number of days covering the experiments. The second stem died on the eleventh day.

approaches the center of the stem. Even when the diameter of the oral and aboral ends is the same and the length of the basal half is

greater or less than that of the apical half, the first apical hydranth is always larger than the basal hydranth. The experiment was repeated on one hundred stems, and the results were always the same in regard to size. The division of the stem by a single ligature into two unit systems increases the number of basal hydranths both in *Tubularia mesembryanthemum* and in *T. crocea*, for in control pieces of equal length one rarely obtains 100 per cent basal hydranths, and after removal of the first one a second rarely ever appears. What, then, causes the increase in number of aboral hydranths when the stem is ligatured? It may be due to the removal of apical inhibition or to wound stimulation, and it may also be due to the establishment of a new polarity after the first hydranth appears. This last supposition is not likely to hold, as the lower half, when cut off below the ligature, retains its original polarity, forming a larger hydranth first on its oral end. It seems more probable that the rearrangement of the cells stimulated by the wound causes an increase in the rate of metabolism and a release of tension, and thus a rejuvenescence of the cells of the piece so that it possesses renewed energy and is thus capable of producing hydranths as long as there is material out of which to make them. As the halves grow shorter by repeated cutting off of the ends, it follows that the new structures must become smaller and smaller. The total number of hydranths on the aboral end may equal and even exceed the number on the oral end, as shown in Figure 11, where the stem is ligatured in two places.

IV. REPEATED REGENERATION AFTER DIVIDING THE STEM INTO THIRDS BY TWO LIGATURES

If the polarity of the basal half of the stem can be reversed by cutting it off from the apical half, it is of interest to see what effect is produced by dividing the stem into thirds (Fig. 10, *A*), thus removing the basal region still farther from the influence of the apical end. In order to prevent any possible confusion of the two ends, the silk used for the distal end differed in color from that used for the proximal ligature; and these colors were used consistently for the two ligatures. Pieces 30–45 mm. in length were employed, so that each section measured from 10 to 15 mm. The results of this experiment on a series of ten stems, running through a period of 9 days, is given

in Figure 11. Here it is evident that after the first set of hydranths appear there is more uniformity in the rate of formation of the two hydranths. Then, too, they are more frequently the same size, and the rhythm in rate of appearance is more marked. How can this be explained? It does not seem possible that this result can be due to the original gradient, for here more hydranths formed on the basal

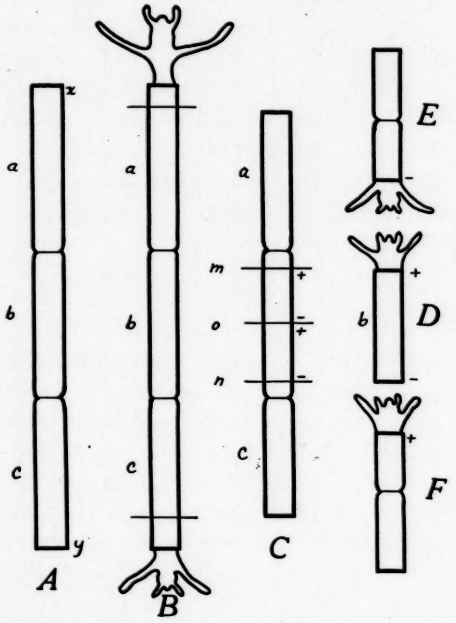

FIG. 10.—Stems divided into thirds (*a*, *b*, *c*) by two ligatures (*A*). *B*, removal of new hydranths. *C*, isolation of the middle piece (*b*) by two cuts at levels *m* and *n*. At *o* the piece *b* is divided in half. In *D* the original polarity of *b* is retained after cutting at levels *m* and *n*. In *E* and *F* hydranths of equal size form at each side of the cut wounds made by the cut in the middle (*o*).

than on the distal end of the stem. It seems more reasonable to assume that repeated wound stimulation and rearrangement of the cells in the basal third have brought about a reawakening of the activity of a part of the stem which has been practically dormant since the stem reached maturity.

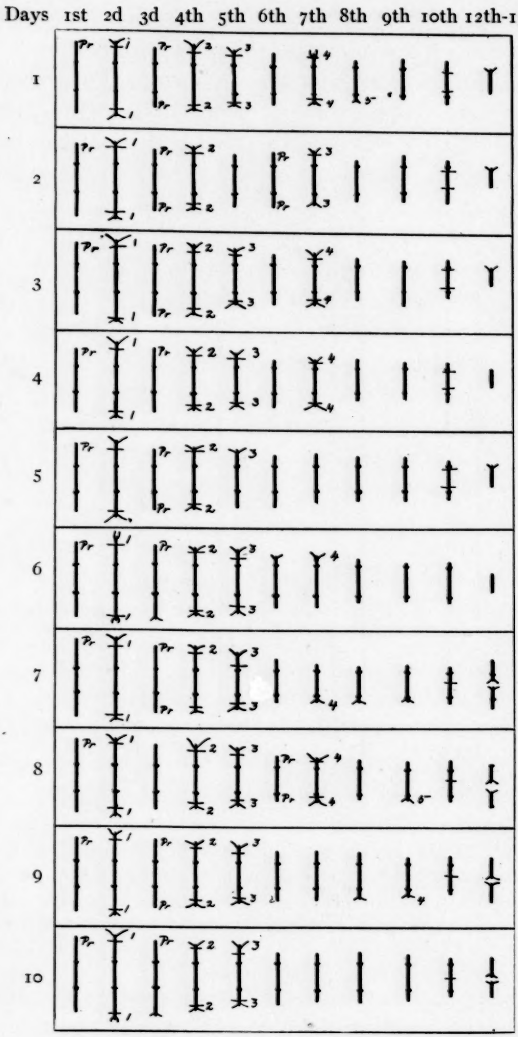

FIG. 11.—Series of hydranths formed during 10 successive days on stems ligatured in two places and cut off repeatedly as in Figure 10.

The middle segment (Fig. 10, *b*) between the two ligatures does not receive this stimulation. When, after several days, it is cut in the middle, as shown in Figure 10, *C*, *o*, the cut surfaces produce new hydranths at the same time and of equal size (*E* and *F*). It is true that the diameter of the two segments is the same and their length equal, but the cut surface of one is aboral and of the other oral. That the entire piece (*b*) may retain its original polarity is seen when it is cut out as shown in Figure 10 *C*, *m–n*, where only an oral hydranth developed (*D*). The development of the two equal hydranths on the middle ends in *E* and *F* seems, undoubtedly, to be due to the stimulus of the wound. The second case can also be explained on the same basis because, being longer, the apical end responded first to the wound and inhibited the response at the basal end. In *E* and *F* there was no opposing influence at the other end; so reconstruction took place at once at both open ends.

V. THE BEHAVIOR OF SHORT BASAL BRANCHED PIECES

When the young polyps of *Tubularia* escape from the gonothecae, they become attached to the rocks upon which the colonies live, and soon develop into new colonies. The directions of stem growth must be more or less governed by external conditions, such as the amount of oxygen and food available; and when the stems have attained a length of several centimeters, the distal free ends bearing hydranths come to occupy the most favorable position while the proximal ends are crowded and tangled and intertwined with the stolon mass and débris. The perisarc of the basal ends becomes hard and brittle, and the coenosarc grows pale and apparently loses much of its irritability. Such regions may be said to be more or less senescent, and therefore would not be expected to display great activity if removed.

Child (1906) has defined regeneration in general as "a return to physiological equilibrium after such equilibrium has been altered by external conditions." What, then, is to be considered equilibrium in the tangled mass of branches, stems, and stolons at the lower end of the colony where it is attached to the substratum? It is almost impossible in this region to distinguish one part from another. *Tubularia mesembryanthemum* branches more than *T. crocea;* therefore it is possible to obtain stems of the Mediterranean species whose bases

are covered with short branches bearing minute hydranths on their tips. It is from such stems that material was selected for this experiment. In Figure 12 a series of ten branched pieces cut from this region is shown. The relative length of the main stem and each branch is indicated in the diagram. The transverse line in each case denotes the removal of the first hydranths formed. When one compares the pieces in this series, it is evident, first of all, that there is a strong tendency to form stolons not only on the basal ends but on the branches as well, and sometimes even on the distal ends. This I have never observed in regions remote from the base. In the second place, no basal hydranths developed on any of them. Third, there is evidence of polarity which may not appear at first but which asserts itself later when the hydranths appear simultaneously, the first time on branch and stem, and the second time on stem only, or on the stem first and later on the branch, the system thus finally establishing a definite polarity. King (1904) studied the behavior of short-branched pieces of *T. crocea* when isolated. She found that when the branch and the main stem were about the same length, hydranths formed on each at the same time; but that when the main piece was longer than the branch, it formed the hydranth first. On the other hand, if the section of the branch was longer than that of the main stem, it developed the hydranth first. This appears to be a case of size or energy dominance. I repeated this experiment in a somewhat modified form, comparing the apical and basal structures with those formed on the branch cut close to the stem (Fig. 12, stem 9). In this series the sections of stem were taken at the base of long stalks which bore one branch only at that level. The branch was cut off close to the main stem, which was 3–10 mm. in length, the branch 1–2 mm. Here 80 per cent formed oral hydranths; none developed on the branches, and only one developed a basal stolon.

A third set of extremely irregular pieces is shown in Figure 13. Here the record was taken 24 hours after the removal of the pieces. Unfortunately this set was lost before its further history was recorded. Even in these exceedingly irregular pieces there is evidence of definite polarity, which is maintained in almost every case; and this may throw some light on the factors at work controlling the direction of growth of young stems. For example, in piece *f*, where

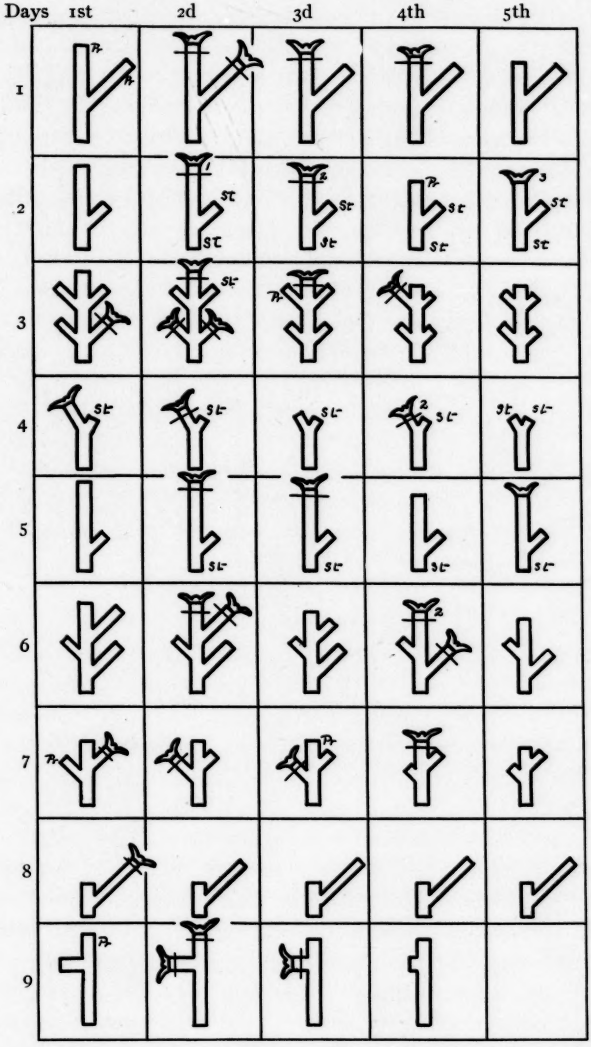

FIG. 12.—Short-branched stems from base of colonies of *T. mesembryanthemum* showing character of structure formed at cut surfaces, and the order of appearance of later structures after removal of the first ones formed.

the main stem is short and the two laterals are longer, it is apparent that these side growths were establishing new systems. This may also be the case in *e* and *m*.

In the last series, Figure 14, six pieces were cut, so that there were two equal branches either at the oral or aboral end of the piece. Forty-eight hours after the operation no aboral hydranths or stolons had developed, although at first the circulation of granules was rapid at every cut surface. Here there is evidence of polarity but no tendency either to form stolons or double structures of any sort. It is not

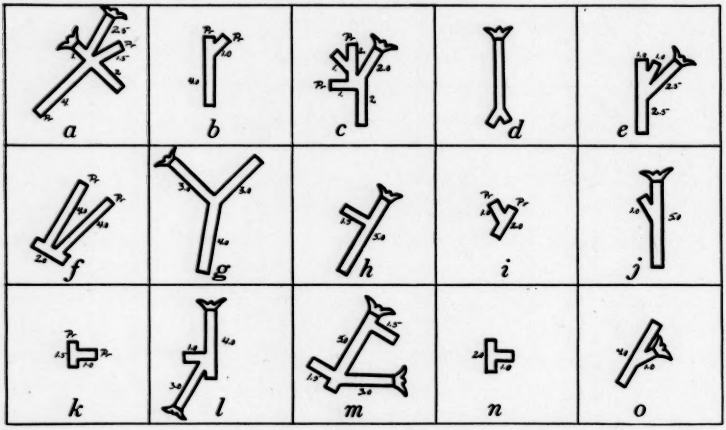

FIG. 13.—Irregular basal pieces from colonies of *T. mesembryanthemum*, showing loss of regularity in the formation of new hydranths on branches. The length of the parts is given in millimeters.

unlikely that the behavior of these irregular pieces may give us a clue to the problem of the origin of the axial gradient. Under conditions of equilibrium it is probable that no secondary or lateral gradients would become established; but in a tangled mass of stolons, branches, and stems, the regions most favorably placed would develop the greatest physiological activity, and so, responding to the conditions most favorable to growth, they would strike out in a direction where they would be less and less hindered in their advance and would finally establish at their distal free ends a region of permanent activity, while their proximal ends, crowded together,

gradually assume a slow metabolic rate and a more or less set or senile condition. Pieces such as those described in the first series have not yet reached this state; they are still plastic and are not yet physiologically differentiated. So when they are isolated from the mass that surrounds them, they develop hydranths and stolons with equal rapidity regardless of size. Lund (1925) has shown by his experiments on *Obelia* that "the phenomena designated organic polarity in organisms represents the operation of a number of quite distinct and yet interrelated mechanisms." Here on the substratum where the development of a hydroid colony takes place, the conditions are so variable that one can account for the comparatively uniform result (the bunched colony) only on the basis of a centrifugally directed growth impulse. In higher animals, as the vertebrates for example, we term this the "law of developmental direction" (Morris, 1914).

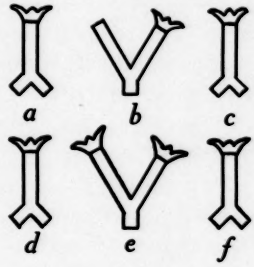

FIG. 14.—Short-branched stems with arms cut in equal lengths. No regularity other than apical regeneration is shown.

In the hydroids *Hydractinia* and *Podocoryne*, where the colony is composed of several different kinds of individuals, nutritive, protective, and reproductive, I (Peebles, 1900) found, after removal of the hydranths, that each individual regenerated its own type of head, showing complete maintenance of differentiation. Pieces of nutritive polyps, when left on the bottom of the dish without changing the water, formed stolons first from the cut ends and later from the sides. These stolons spread out in all directions until it was impossible to distinguish the original stem from which they started. Very soon new hydranths formed, but these were always nutritive in structure. Pieces of the protective polyps were isolated in the same way, and stolons grew out from them; but these stolons spread very slowly, and new individuals did not form from them. Tripp (1928) has also studied the formation of colonies of *Podocoryne* by the same method. He found that the presence or absence of food played a great part; but his experiments do not throw any light on the cause of the direction of growth of the stolons, which is in all probability determined

by differences in surface tension. He considers the formation of the stolons a type of budding, while the hydranths represent a regenerative process. In these hydroids it seems likely that the stolon development resembles the growth of cells in a tissue culture and is largely influenced by the medium by which they are surrounded, by the oxygen supply, and possibly by the bacteria present. In *Tubularia* this remolding of the body form cannot take place on account of the surrounding perisarc, the outgrowth is limited to the ends of the isolated pieces, and branching of the stolons does not take place from the distal end until hydranth formation has ceased; but long stolons frequently form on the proximal end, and, after growing out for some distance, form hydranths on their free ends. This resembles the process of budding and is very different from ordinary oral hydranth building.

The question then arises as to the origin of the branches in the lower ends of the stems. Hyman (1926) considers branches in *Tubularia* an indication that the dominance of the terminal hydranth does not extend very far in a proximal direction. This does not seem to explain the irregular groups of branches so frequently found in *Tubularia mesembryanthemum*, which have undoubtedly arisen under abnormal conditions, for they are gnarled and twisted like the branches of weather-beaten trees. It is evident that they have not arisen from injury to the main stem. Morgan (1904) attempted to produce branches by artificial means; and I have tried repeatedly to cause some outgrowth from the stem by wounding the perisarc, but the results have been negative. It seems, then, highly probable that branches have arisen through conditions which changed the direction of growth; or they may have arisen through a coalescence of very young pieces. They have every appearance of abnormal development; and their behavior, when isolated, supports this hypothesis.

VI. REGENERATION OF YOUNG AND OLD STEMS COMPARED

The experiments of Hyman (1926) on *Tubularia* have demonstrated that pieces from smaller, i.e., younger, stems produced relatively larger hydranths in a shorter time than pieces of equal length from larger stems. Przibram (1919) says the rate of regeneration is always more rapid the younger the animal.

The size of the piece is not always an indication of its age. Frequently one finds small slender stems in which growth has evidently been retarded through adverse conditions. They seem to contain very little coenosarc and are usually paler in color than the other stems. These pieces, when isolated, are slow in forming new hydranths and rarely develop any aboral structures. In Table III the results from a number of mass experiments are given. The percentage of basal hydranths is much greater on the old stems than on the younger ones.

TABLE III

COMPARISON OF APICAL AND BASAL STRUCTURES FORMED ON YOUNG AND OLD STEMS

Young Stems					Old Stems				
Average Length in Mm	No. of Pieces	Percentage of Hydranths		Percentage of Stolons	Average Length in Mm	No. of Pieces	Percentage of Hydranths		Percentage of Stolons
		Apical	Basal				Apical	Basal	
20.....	10	70	40	0	12.....	10	100	70	0
18.....	8	100	25	0	12.....	12	100	66	0
15.....	28	100	35	0	22.....	20	100	40	0
12.....	12	100	0	0	15.....	50	100	50	0
15.....	25	100	33	0	18.....	13	100	30	0
Total..	83	94	26.6	0	Total..	105	100	51	0

No conclusion can be drawn in regard to the tendency to form stolons on either young or old stems, as none at all appeared. The failure to form 100 per cent apical hydranths on the younger stems was undoubtedly due to conditions of depression at the time of the experiment. This may also account for the absence of stolons.

VII. THE RELATION OF THE LENGTH OF THE PRIMORDIUM TO THE VOLUME OF THE STEM

It has been demonstrated by Child (1907*b*) that there is a definite relation in proportion between the length of the piece and the length of the primordium; also, that there is a variation in the proportion of tentacle length at the time when the two rows are laid down. I have observed that there is great variation in the length of the complete primordium (Fig. 15, *B*, *x*–*y*). The relation of the primordium length to volume was studied in the following manner: A stem 25–30 mm.

in length was cut in half (Fig. 15, *A*, *x* and *y*); the length and diameter of each cylindrical half were measured; and as soon as the primordium (*pr*) was laid down, its length was measured (Fig. 15, *B* and *C*). The volume of each half-stem was then computed and compared with the length of the primordium. It was found that in half-stems the length of the primordium was always greater on the distal half (*x*) than on the proximal half (*y*) and that this difference was maintained even if the volume of *x* was less than the volume of *y*. For example, in Figure 15, *B* and *C*, two stems are shown; the dimensions are given for diameter, length of piece, and length of primordium. In *B*, the volume of the apical half (*x*) is 9.5426 cu. mm., of the basal half (*y*) it is 3.8484 cu. mm., and in *C* the volume of the apical half (*x*) is 2.2619 and of the basal half (*y*) it is 5.0893 cu. mm.; yet both primordia in the distal halves are longer than the primordia in the proximal halves. Measurement of many pieces shows that the distal half always produces a longer primordium than the proximal half. Child (1907) has observed this, and also that the primordia of basal hydranths are always shorter than those of apical hydranths. The length of the primordium, while it is definitely related to the size of the hydranth which develops from it, is not an indication so much of the volume of the piece as it is of the position that it occupies.

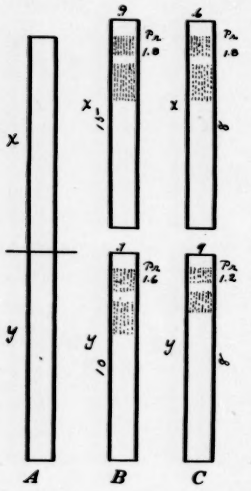

FIG. 15.—Showing the relation of the length of the primordium to the volume of half-stems. *A* shows stem cut into halves *x* and *y*. *B* and *C* indicate the dimensions in millimeters.

VIII. GROWTH IN LENGTH OF HYDRANTH STALKS

When the new hydranth emerges from the cut end of a stem, it is carried out by the rapid growth of the region immediately below it. This region varies in length—sometimes extending 7–10 mm.; at others not more than 4–5 mm. Also, there is great variation in the length of the stalk of the basal hydranth. In Table IV a series of

measurements is given for the stalks of oral and aboral hydranths on the distal (x) and proximal (y) halves of the stem for three consecutive days (Fig. 7). The average growth in length of the hydranth stalks was greater in the proximal halves of the stem than in the distal halves, and in basal hydranths greater than in apical hydranths; but there is great individual variation in stems as the

TABLE IV

GROWTH IN LENGTH OF THE STALKS OF NEW HYDRANTHS FORMING ON THE ENDS OF PROXIMAL AND DISTAL HALVES OF STEMS

No. of Stem		Length in Millimeters					
		First Day		Second Day		Third Day	
		Apical Stalk	Basal Stalk	Apical Stalk	Basal Stalk	Apical Stalk	Basal Stalk
1	x	2.0	0.0	3.0	2.0	4.0	2.0
	y	7.0	3.5	10.0	7.0	13.0	10.0
2	x	2.5	2.0	5.0	3.5	5.0	2.0
	y	0.0	0.0	6.0	2.0	Dis.	Dis.
3	x	3.0	0.0	4.0	3.0	4.0	3.0
	y	4.0	3.0	4.0	4.5	8.0	4.0
4	x	4.0	2.0	6.0	4.0	6.0	4.0
	y	4.0	4.0	5.0	5.5	Dis.	Dis.
5	x	8.0	0.0	6.0	2.5	6.0	0.0
	y	4.0	2.0	4.0	2.5	Dis.	Dis.
6	x	5.0	1.0	5.0	2.5	5.0	4.0
	y	4.0	0.0	5.0	1.5	6.0	Dis.
7	x	3.0	0.0	3.0	0.0	4.0	Dis.
	y	3.0	2.5	3.0	2.5	2.5	2.5
8	x	0.0	3.0	4.5	1.5	4.0	0.0
	y	4.5	1.5	3.0	0.0	Dis.	Dis.
9	x	5.0	2.5	5.0	3.0	Dis.	Dis.
	y	3.0	2.0	3.0	2.5	Dis.	Dis.
10	x	3.0	0.0	4.0	0.0	5.0	3.0
	y	4.0	2.0	6.0	2.5	Dis.	Dis.
	Av. x	3.5	3.5	4.5	4.9	4.7	6.0
	Av. y	1.5	2.5	2.2	3.5	2.3	5.5

table shows. But the basal and distal hydranths begin to disintegrate on the third day, this disintegration becoming more rapid on the basal ends.

DISCUSSION

The problems connected with the process of reconstitution of the individual from an isolated part are by no means solved. The growth impulse inherited by every organism expresses itself in patterns

which are modified by the surrounding universe. One must consider the physical effects of cells upon one another through pressure and tension; also, the chemical effects of the products of metabolism, the exchange of gases, and the formation of definite substances which either promote or hinder the inherent force residing in each cell. So far as we know, there is no embryonic tissue in the hydroid stem corresponding to the meristem or cambium of plants. All the cells seem equally endowed with "this protoplasmic complex of inheritance, the growth impulse" (Greene, 1928). The growing tip of *Tubularia* is not distinguishable in structure from the rest of the stem; but it becomes, upon stimulation, a veritable storehouse of energy from which supplies are continually given off.

A stem of *Tubularia* is potentially capable of independent growth: it forms a new hydranth, frequently a stolon on the basal end, and becomes attached to the substratum. Stolon formation may be interpreted as the expression of the pure growth impulse, while in hydranth formation differentiation sets in at once. The first is primitive and more likely to occur in young pieces. The reason why we do not get stolons on the apical end is that here is a specialized region, although stolons may be produced even at this level by subjecting the pieces to changes in environment through the action of chemicals and through dilution of the sea water (Child, 1907*d*). While the hydranth is more elaborate in structure than the stolon, it is produced largely through working over of cells already present; and certain chemical and physical conditions must be met in order to produce both stolon and hydranths. The growth of a stolon is more embryonic in nature and is the expression of a primitive process.

The question that arises first is, "Why does the apical hydranth always form first, and why is it always larger than the basal one?" There are several reasons for this. First, as has already been shown by many investigators, this is the region of greatest metabolic activity. Second, it is the region of greatest tension, and the release of this tension brings about a series of changes in cell relation. Third, the normal direction of growth is away from the base of the colony, and this growth is more or less hindered while the old hydranth is present but is released when the hydranth is cut off. And last, the youngest cells are here, and they are able to adapt themselves more rapidly

than older ones to any great change in the surroundings. The larger size of the apical hydranth is explained, first, by the greater metabolic rate in this region and, second, by the extent of wound surface rather than by the volume of the piece.

The next question that confronts us is whether or not there is real apical dominance in isolated pieces of the stem or whether the delay in the formation of the basal hydranth is due to some other factor. It is certainly true that the aboral hydranth is slower in starting to form; but as soon as the primordium is once laid down, the hydranth is complete and emerges as rapidly as the oral one. This early delay is perhaps due to the condition of the cells in this region. They have lost their growth impulse because they have remained so long at the base of the stem, where conditions are in favor of its suppression rather than its furtherance. The cells here have actually aged, and it therefore takes time for them to overcome their inertia; but as soon as they are once started, they accomplish the formation of a hydranth as rapidly as the apical cells.

Basal ends, when subjected to continuous wound stimulation, may form a larger number of hydranths than apical ends if they are cut off by a ligature from the influence of the apical region. In the stems of *Tubularia* apical dominance plays a much smaller rôle than in plants where regions of varying length below the apex are held in abeyance as long as the apical region is in tact. The two cases are therefore not altogether comparable. On the other hand, the inhibitive influence of the apical end of *Tubularia* may be due to the influence of a growth-inhibiting substance set free as a by-product of metabolism. The circulation of this substance in the stem may delay growth at the basal end, and this may explain the formation of hydranths sooner on the basal ends of stems that have been ligatured.

Embryonic growth in general is relatively more rapid in apical regions and in a cephalad direction, and less rapid in a basal direction. It is scarcely possible to compare the processes; yet the higher rate of metabolic activity at the distal end would perhaps warrant the expectation of greater growth in length in that region. But, on the other hand, stolons are more frequent on basal ends, and their growth in length is much more rapid than the growth of hydranth stalks.

Many years ago Morgan (1904) pointed out that individual differences in stems play an important rôle in the behavior of isolated pieces. These differences we now attribute to chemical and physical variations in constitution of the individual cells. We speak of "physiological activity" and "chemical energy" now, where the earlier workers spoke of the "organizing principle" and "formative organization." Child (1925) has shown how it is possible experimentally to reduce or eliminate polarity and to reverse or intensify it. Therefore it appears that much of the behavior of pieces of *Tubularia* stems may be traced directly to external influences but that the growth impulse inherent in the living matter, which is held more or less in abeyance in the complete system, asserts itself immediately when there is a tension of the cells or the stimulation of a cut surface. This impulse has become even more suppressed or reduced at the base of the stem, so that it takes a little time for it to assert itself or to respond to a change; but as soon as the basal end becomes adjusted it seems capable of organ-building for as long a period as the distal end, and frequently to produce more structures.

In recent publications Child (1927, 1928, and 1929*a*) has laid emphasis on the transmissive rather than the transportative side of dominance and subordination, and has developed an exceedingly alluring series of advances along this line from the lowest to the highest forms where nervous control is fully established. On the other hand, he does not forget the important development of transportative relations terminating in the elaborate rôle of hormones. There is reason to believe that further investigations in the field of chemical stimulation will throw light on the transportative side. Since such an organism as *Corymorpha* (Child, 1929*a*) establishes a new axis of growth and is stimulated to activity by the mere opening of its wall, there must be profound changes produced by the contact of the open surface with the salts of the water and by the release of the tension caused by cell contact.

There is evidence that this contact of the open surface with the sea water is a factor that must be given consideration. Driesch (1899) pointed out many years ago that the stimulus of the water was significant. Child (1925) found that regeneration and growth are greatly modified by dilution of the sea water.

Loeb's (1892) original hypothesis of the organ-forming substance influenced the early work, but Morgan (1901*a*) threw doubt on the theory by experiments dealing with the origin and fate of the red granules that are always seen circulating in the ends of the stems. He showed that these granules, instead of being organ-building substances, were products of disintegrating cells. Child has abandoned all idea of such a substance and has substituted the theory of the axial gradient maintaining that hydranths form where the metabolic activity is greatest and that the rate of development at the various levels is controlled by the position of the piece in relation to this gradient.

Spemann (1918–29) in his brilliant investigations on development and growth has drawn attention to the part played by "organizers," and Child (1929) has pointed out that the active regions of the stem of *Tubularia* act as organizers to a greater or less degree.

There is no evidence that a growth-promoting substance exists in *Tubularia*, but the results of wounding the stem in *Corymorpha* (Child, 1929*a*) and of repeated cutting-off of hydranths in *Tubularia* point to the supposition that some substance is formed as a result of exposure to sea water and that this substance affects the building of new structures.

In conclusion, one is forced to fall back upon the supposition that the extent of growth and the character of structures regenerated at a given time are largely influenced by the conditions to which the organism has been subjected prior to the date of observation and experimentation. The growth impulse is furnished to each cell by the genes which are handed down to it from former generations; but the cells at the apical end of the stem, although they contain the same genes as the cells at the basal end, react more rapidly to changes in the environment such as release of tension and the exposure of the cut surface to sea water. By repeated removal of basal parts, the nature of the cytoplasm can be modified, and it loses its set condition. Such modification results normally during the breeding season, when the cytoplasm becomes rejuvenated and it then exhibits a degree of development impossible at other times. The solution of many of the problems rests in tracing the history of the individual back to its beginning. From the time the young

polyp first settles on the rocks, it is subjected to conditions of varying influence; the system developing from it is necessarily an expression of these forces resulting in the establishment of a growth habit. Dividing stems by one or more ligatures and repeatedly removing new hydranths as soon as formed, proves that the more one experiments with the organism the more plastic it becomes and the nearer it approaches a state of adaptability resulting in the formation of new parts until its supply of energy is exhausted.

SUMMARY

1. Hydranth formation in *Tubularia* begins in cut ends many hours before the appearance of the primordium. This is demonstrated by the removal of short distal ends at definite periods after the original hydranth has been cut off. If the tip is removed 2–4 hours after the isolation of the stem, the short piece reorganizes itself and develops a small hydranth. By the sixth hour head formation has set in, and some of the short pieces form as large hydranths as if they had remained on the stem. At the end of the thirteenth to fifteenth hours the hydranth is so far advanced that reorganization is rare.

2. Short basal ends, if removed from the stem after 6 hours, form more aboral than oral hydranths. If left a longer time on the stem under the influence of the apical end, they develop more hydranths on their oral ends, although basal development is not entirely suppressed.

3. The changes which take place at both the distal and proximal ends of long isolated stems affect all levels of the piece, causing a stimulus which is evidenced by the production of a large percentage of basal hydranths. The operated stems formed a larger number of basals at all levels than the controls which were cut into the same number of pieces.

4. Half-stems, when divided into a number of pieces, show the influence of wound stimulation and apical dominance if each half is allowed to begin development before it is cut into small pieces.

5. After single and double ligatures the first hydranths that appear on the cut ends differ greatly in size, the larger ones always forming on the oral ends. After repeated removal of the newly

formed hydranths, the successive ones become more and more nearly the same size and appear simultaneously.

6. In ligatured stems there is indication of rhythm in the development of successive hydranths, the first set appearing after 48 hours, the second after 24 hours, and the third not emerging until the seventy-second hour or later. A large percentage shows this speeding-up of the developmental process after reconstruction has once set in.

7. The formation of a new hydranth on the oral end of a short-branched stem does not inhibit the development, or influence the size, of the hydranths forming on the branches. The clusters of small branches at the base of the stem of *Tubularia mesembryanthemum*, and the more scattered branches of *T. crocea*, apparently represent abnormal growth caused by external conditions; and they indicate in their behavior, when isolated, that they were setting up for themselves an independent direction of growth at the time of removal from the mass.

8. Size is not a safe criterion in the determination of the age of a stem. The terms "young" and "old" cannot be applied indiscriminately to short and long stems, and it is not always safe to regard those of small diameter as young stems. This is demonstrated in the behavior of isolated pieces of the same length and same diameter. *Physiologically* young stems have a very delicate perisarc and are usually pinker than the older ones, and they produce hydranths with fewer tentacles. The older stems produce a larger percentage of basal hydranths. At the reproductive season all stems become *physiologically* young, and at this time stolon formation is more frequent.

9. The length of the apical primordium in half-stems is an expression of the level of the piece rather than of its volume. The oral primordium on the distal half of the stem is always longer than that of the proximal half, even if the volume of the lower half is greater than that of the upper half.

10. In half-stems the growth in length of new hydranth stalks is generally greater on the ends of the proximal than distal halves, and basal stalks average greater increase in growth than those on distal hydranths.

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SEPARATION OF ANTERIOR-LOBE SUBSTANCES AND STUDY OF THEIR INDIVIDUAL EFFECTS[1]

(Six plates and two text figures)

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THAT the anterior lobe of the hypophysis secretes substances which influence and regulate normal growth and reproduction in mammals has been demonstrated by many investigators. This has been done in various ways: by hypophysectomy, transplants, and injection of extracts from this gland. Since the literature concerning this subject has been reviewed so frequently only a few experiments bearing directly upon the effects of extracts will be discussed in this paper.

Evans and Long (1922) proved without doubt that an alkaline extract of the anterior lobe contained a growth-promoting substance. If this extract were injected daily over a long period of time, giant rats resulted. Associated with the injection of this particular extract was noticed a disturbance of the oestral cycle of the rat. Examination of the vaginal smear showed that the first oestrus occurred later in the treated animals and after that ovulation was infrequent if it occurred at all. The ovary of the injected animals contained atretic corpora lutea, that is, follicles whose theca and granulosa layers had formed lutein cells inclosing the ovum.

This same phenomenon of luteinization of the follicles has been described by Walker (1925), Brouha (1928), Botschkareff (1929), Courrier (1929), Claus (1929), Grueter (1929), Hewitt (1929), Johnson and Sayles (1929), Zondek (1929), Friedman (1929), and Wiesner (1930), after injections into rats, mice, and rabbits of saline, weakly acid, or alkaline extracts of the anterior lobe of the pituitary body or of urine from pregnant women.

[1] The expense of these investigations was borne in part by funds supplied to Professor Guyer by the Committee on Maternal Health.

A condition of overgrowth more nearly resembling cases of human acromegaly was produced experimentally by Putnam, Benedict, and Teel (1929) in dogs. One animal, which received daily for fourteen months an aqueous extract of beef anterior lobe (made according to a modification of Evans' method), became about twice as heavy as its litter-mate control. Hypertrophy of the genitalia of the dog suggested that the hormone which causes precocious sexual maturity might also be present in the extract injected.

Bellerby (1929) produced spontaneous ovulation in rabbits by injecting intravenously an acetic acid extract of anterior lobe. This acid extract was prepared for injection by shaking with kaolin, neutralizing and filtering. Urine of pregnant women is likewise effective, according to Friedman (1929), when injected intravenously but not intraperitoneally. Jares (1930) recently confirmed Friedman's work but could not induce ovulation using Bellerby's method. He could, however, if he omitted the kaolin treatment.

From the urine of pregnant women, which has been acidified with acetic acid, concentrated, shaken thoroughly with ether to remove oestrin and dialyzed, Aschheim and Zondek (1928) prepared an extract which they have named "Prolan." This stimulates the animals to sexual maturity and follicular development in a short time, but if injected in large doses for a longer period causes luteinization of the follicles. These results have been confirmed by Biedl, Botschkareff, Brouha, Ehrhardt (all 1929), and others.

It has, then, been demonstrated by means of extracts that the anterior lobe of the hypophysis contains a substance which materially affects growth by producing gigantism and the condition known as acromegaly. According to Evans and Long (1922), the growth-promoting extract at the same time causes luteinization and inhibition of oestrus. Putnam, Benedict, and Teel (1929) find that their growth-promoting extract stimulates sexual maturity and follicular development. Extracts from urine of pregnancy when injected in certain amounts stimulates the ovaries, but in larger amounts it inhibits ovulation and luteinizes the follicles.

The question arises of how many hormones there are in the anterior lobe of the hypophysis. Are there three separate substances, one affecting growth, a second stimulating follicular development,

and a third inhibiting ovulation by stimulating the formation of atretic follicles; or are the first and third one hormone as it would seem from Evans and Long's (1922) experiments; or are the first and second the same as Putnam, Benedict, and Teel's (1929) work would indicate; or are the second and third different degrees of action of the same hormone as the "Prolan" of Zondek (1929) would suggest? These questions have not been satisfactorily answered, nor can they be until the hormones have been purified to the state such as thyroxin and insulin have been at the present time.

The object of the experiments described in this paper was to attempt if possible the separation and purification of the hormones of the anterior lobe of the hypophysis and to determine their physiological effects upon rats and mice.

TECHNICAL PROCEDURE

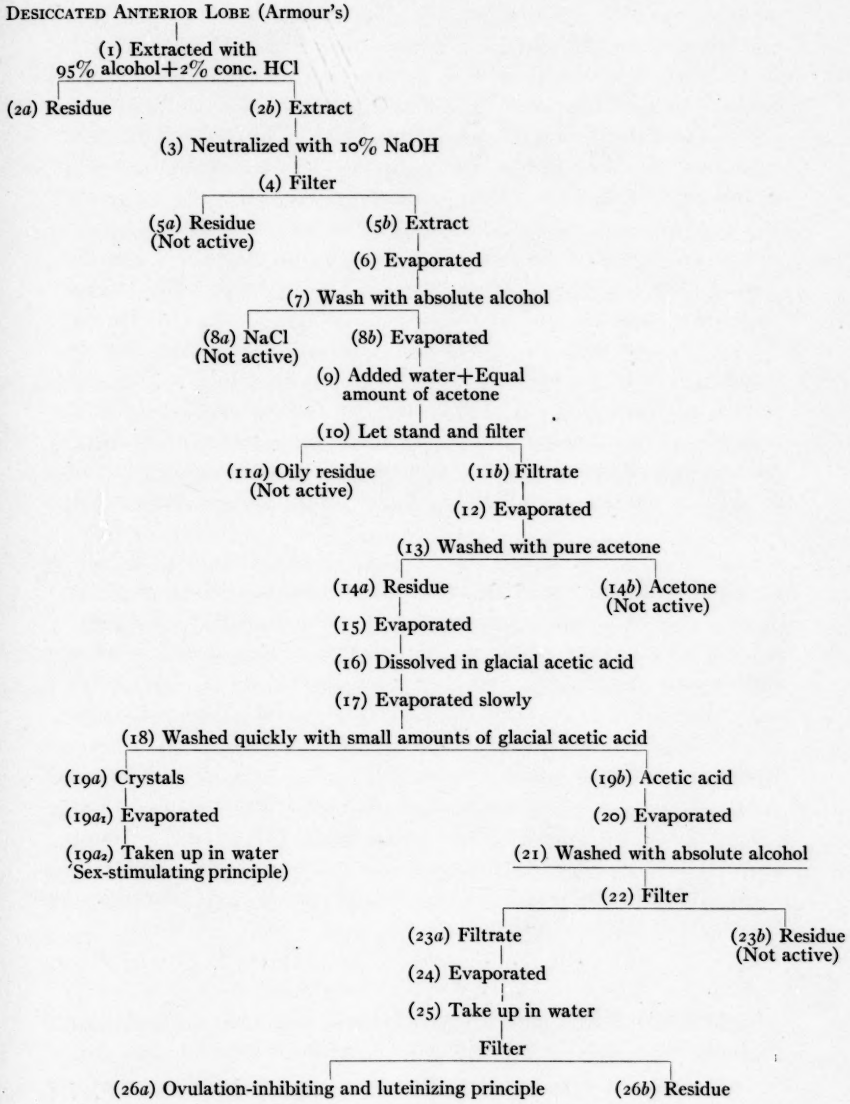
In the spring of 1928 several attempts were made to extract Armour's desiccated anterior lobe with water and normal saline in order to obtain a product which would cause inhibition of oestrum in the rat. This being unsuccessful, an acid alcoholic extraction was made according to Hisaw's earlier method for extracting corpus luteum in which the salt of neutralization was eliminated by dialysis. It was found that if a small amount of this crude extract were injected, the ovary was stimulated to greater follicular activity, while if in larger amounts, inhibition of ovulation and luteinization of follicles occurred.

The improved technique of Fevold, Hisaw, and Meyer (1930) for the separation of the hormones of corpus luteum was then followed with some modifications. An outline of this method is graphically presented in Chart I; also a detailed description follows.

Fifty grams of desiccated anterior lobe is extracted with 500 cc. of acid alcohol (2 cc. concentrated HCl to 98 cc. 95 per cent alcohol). This is stirred several times, then allowed to stand at room temperature and extracted for about two hours or until the alcohol is colored dark brown and the solid material has settled to the bottom. The extract is then turned off and more acid alcohol is added and reextracted until the alcohol no longer becomes colored. The various extractions are usually completed within eight to sixteen hours and are then neutralized to litmus with 10 per cent NaOH. At this point

CHART I

METHOD OF PREPARING ANTERIOR-LOBE CRYSTALS



a white flocculant precipitate settles out which is filtered off and discarded. The filtrate is evaporated in a drying oven at 37° C. to semidryness. By washing at step 7 with absolute (99 per cent) alcohol most of the hormone is taken up by the alcohol while the NaCl is left behind. In order to be sure that all the active material is obtained acid alcohol is again added to the residue (8*a*) and steps 3–7 repeated until the salt left is almost white. The absolute alcoholic washings are combined and evaporated. To this about 25 cc. of water is added and emulsified, and followed by an equal volume of acetone. The precipitated phosphatides are oily and rise to the top, but after standing a few minutes can easily be filtered off and discarded. The filtrate (11*b*) is evaporated to semidryness and washed with pure acetone to remove any remaining fats. The gummy residue (14*a*) in this instance contains the active material. Any remaining acetone is evaporated from the residue which is then dissolved in about 75 cc. of glacial acetic acid. The acetic acid is allowed to evaporate slowly either at room temperature or at 37° C. As this evaporates the follicular stimulating hormone crystallizes out in combination with NaCl in a very characteristic form (Plate I, Fig. 1).

Since the crystals at this stage are not as soluble in glacial acetic as is the brown material, they can be purified by quickly washing them with small amounts of acid until no brown substance is left; 19*b* will still contain some crystals, so steps 17 and 18 are repeated until no more crystals can be seen with the aid of the microscope when the acid has evaporated. The crystals are combined with 19*a* and for injection, all acetic acid is evaporated off, and the residue dissolved at once in distilled water.

After the last recrystallization from 19*b* this fraction is evaporated and extracted with absolute alcohol. A slight precipitate will form which is filtered off and discarded. The filtrate is evaporated and dissolved in distilled water for injection. This may again be filtered if any of the material does not dissolve.

TEST ANIMALS

Both immature and mature female albino mice and rats have been used as test animals for injections of the anterior-lobe extracts. Im-

mature male mice have also received injections. These animals were taken from the laboratory colony, which has been inbred for a number of years and hence standardized. They were in the best possible nutritional condition, having been fed a balanced diet supplemented by green food daily.

PHYSIOLOGICAL EFFECTS OF THE CRYSTALLINE EXTRACT

Immature female mice.—For injection, crystals from step 19*a* were dissolved in distilled water in such proportion that 1 cc. represented 10 grams of fresh anterior-lobe tissue, or 2 grams of the desiccated material. One-tenth of a cubic centimeter is then equivalent to 1 gram of fresh material or $\frac{1}{5}$ gram of desiccated anterior lobe. In the first experiment (Table I) eighteen immature mice from 17 to 21 days old were injected with 0.1 cc. of the crystals. The dissolved crystals were injected once a day until the vagina opened. The opening of the vagina and the vaginal smear were taken as one criterion of maturity of the animal. In order to make a histological examination the injected animals and the controls were killed the day of maturity and the ovary, uterus, vagina, and pituitary body were fixed in Bouin's fluid. As a result of the injections of crystals immature female mice were sexually mature at an average of 21.8 days while observations on a large number of normal animals showed the average date of maturity of non-injected mice to be 30.5 days. Upon microscopical examination the ovaries of the injected animals (Plate II, Fig. 5) showed an increase in size with large mature follicles. The uterus was enlarged, owing to an increase in the thickness of the muscular and mucosa layers. The lumen of the uterus was also considerably enlarged. In contrast to the immature mice, the cells of the epithelial layer of the uterus were columnar in type rather than cuboidal. The vaginal epithelium shows a response to the injection of anterior-lobe crystals which is equally characteristic of maturity as are the changes in the rest of the genital tract. Plate II, Figure 7, shows proliferation of the cells of the mucosa which is quite different from the condition of the immature mouse shown in Plate III, Figure 10. In fact, the opening of the vagina and establishing of the lumen, and the picture of the ovary and uterus, is identical with that seen at normal maturity.

Varying the dosage.—Increasing the amount of the crystalline extract injected into mice as seen by Table II does not shorten the

TABLE I

Mouse No.	Dosage Daily	Started: Age in Days	Maturity: Age in Days	Remarks
	cc.			
1	0.1	18	22	
Control				Both killed at 23 days
2	0.1	18	23	
Control				Both killed at 23 days
A	0.1	18	20	
Control				Both killed at 20 days
C^1	0.1	18	20	
C^2	0.1	18	20	
Control				All killed at 20 days
D^1	0.1	21	22	
D^2	0.1	21	23	
Control				All killed at 23 days
E^1	0.1	18	21	
E^2	.1	18	20	
E^3	0.1	18	21	
Control				All killed at 22 days
H	0.1	18	22	
I	0.1	18	22	
Control				All killed at 22 days
J	0.1	18	22	
Control				All killed at 22 days
K	0.1	18	20	
Control				All killed at 22 days
L^1	0.1	17	22	
L^2	0.1	17	22	
Control				All killed at 22 days
I_{93}	0.1	18	25	
Control				All killed at 25 days
I_{104}	0.1	18	25	
Control				All killed at 25 days
Average	0.1	18.1	21.8	Matured in 3.7 days

time of maturity. The average time would appear, on the contrary, to be lengthened somewhat. This may only appear so, however,

because of the smaller number (eleven) of animals used. It will be seen from all these experiments that there are individual differences in mice which may be due to their variability in weights at the same age.

Smaller doses of crystals are just as potent as the larger amounts, as shown by Table III. In fact, in one case (I_{86}) only two injections

TABLE II

Mouse No.	Dosage Daily	Started: Age in Days	Maturity: Age in Days	Remarks
	cc.			
P_1	0.25	16	22	
P_2	.25	16	22	
I_{13}	0.25	17	24	
Average	0.25	16.3	22.6	
I_{46}	0.2	18	23	
Control			31	
I_{103}	0.2	17	25	
Control				Both killed at 25 days
I_{106}	0.2	18	20	
I_{107}	0.2	18	20	
Control				All killed at 21 days
I_{108}	0.2	18	23	
I_{109}	0.2	18	23	
Control				All killed at 23 days
I_{110}	0.2	18	22	
I_{111}	.2	18	23	
Control	0.2			All killed at 23 days
Average	0.2	17.9	22.3	

of 0.01 cc. each were administered when the vagina opened. The crystals used in this instance had been dried and weighed so that a total of 0.026 mg. of crystals, equivalent to 0.2 grams of fresh anterior-lobe tissue or 0.04 grams of desiccated substance, was injected.

Immature male mice.—That immature male mice can be stimulated to precocious development by injections of crystalline extract of anterior lobe has been demonstrated by the fact that 21-day-old males may copulate with an injected litter-mate female. Normally male mice mature between 40 and 50 days of age, and never in our

experience has an uninjected male copulated as young as 30 days old, even when kept as was usually true in the same cage with injected mature females. Daily vaginal smears were taken from the female, and copulation would have been detected by the presence of spermatozoa or by pregnancy. The size of the accessory glands of the genital tract in injected males are also noticeably larger than those of uninjected animals.

Castrates.—Crystals did not cause precocious opening of the vagina after ten injections in immature female mice which had been castrated previous to the injections, nor did the crystals bring an

TABLE III

Mouse No.	Dosage Daily	Dosage Total	Started: Age in Days	Maturity: Age in Days
	cc.	cc.		
I_{86}	0.01	0.02	18	19
I_{88}	.01	.05	20	25
I_{84}	.02	.10	18	22
I_{85}	.03	.12	18	22
I_{87}	0.04	0.20	18	21
Average	0.022	0.098	18.4	21.8

adult castrate rat into heat. Since the injection of oestrin will open the vagina of an immature castrate mouse and will bring an adult castrate rat into heat, it appears that oestrin acts directly upon the vagina and uterus, while the anterior-lobe substance would seem to act first upon the ovary and indirectly upon the uterus and vagina. These experiments would also indicate that the crystalline extract of anterior lobe contains little or no oestrin.

Immature and adult rats.—Because of the lengthy procedure necessary to obtain crystals and also because the material is very expensive, mice were used chiefly as experimental animals, since it takes a larger quantity and a longer time of injection to produce the same results in rats. However, a series of five immature rats injected from the time they were 25 days old with an equivalent of 30 grams of fresh tissue per day matured in $5\frac{1}{2}$ days. The normal time of maturity for rats, according to Long and Evans (1922) is from 34 to 109 days of age. This will be repeated again, using smaller doses of the crystalline extract to determine a minimum rat unit.

Plate III, Figure 11, shows a cross-section of an ovary of an adult female rat which had been injected with the follicular stimulating hormone. The uterus was greatly distended with liquid and the vaginal epithelium was much proliferated and cornified. This animal had been in heat for 6 days. Eleven adult rats injected with crystals equivalent to 3–5 grams of fresh material daily exhibited an exaggerated lengthening of the oestral period and practically no true dioestrum, that is, no time when scales or nucleated cells were not found in the smear. If, however, these animals are killed after ovulation many normal corpora lutea will be found in the ovary giving it a typical mulberry appearance. No atretic corpora lutea were present.

TABLE IV

Mouse No.	Dosage Daily	Started: Age in Days	Maturity: Age in Days	Remarks
	cc.			
I_{15}	0.5	17	22	
I_{17}	.5	17	24	
I_{21}	0.5	17	21	
Control				All killed at 28 days
Average	0.5	17	22.3	

Crystals and growth hormone.—An alkaline extract of desiccated anterior lobe was made, according to the method of Evans and Simpson (1928). Then from the residue, an acid-alcohol extract was made as already described, and crystals of the same appearance as those from the intact anterior lobe were obtained. Results as shown in Table IV indicate likewise that the crystals still contain the active material. The substance may be a bit less active but too few animals were used to rule out individual variations, hence no conclusion as to the amount of active substance present can be drawn.

An acid-alcohol extract was first taken from the desiccated anterior lobe and the residue was then extracted for growth hormone by the alkaline method. Text Figure 1 shows that the injected rats are very little heavier than the controls after 182 daily injections of $\frac{1}{4}$–$\frac{1}{2}$ cc. of growth hormone. Evans (1922), however, ran his experiments over a period of 200–400 days and found the greatest increase in growth during the last third of this period. So, in spite of the indi-

cation that the acid had inactivated the growth hormone, injections must be made for a longer time before conclusions can be drawn.

Control experiments.—As a control experiment yeast submitted to the same method of extraction yielded crystals which were different in appearance and which produced none of the physiological changes that have been described for anterior-lobe substance. Fevold, Hisaw, and Meyer (1930), moreover, obtained an entirely different type of

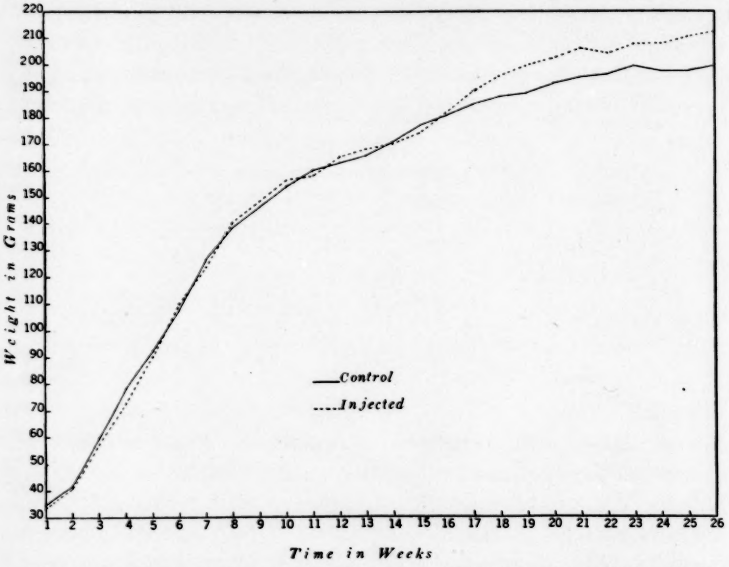


FIG. 1.—Average growth-curves of immature male and female rats injected with alkaline extract made after acid extract had been removed. Controls received no injection.

crystal from corpus luteum substance although the procedure described in this paper is essentially the same as theirs.

SOURCES OF ANTERIOR-LOBE CRYSTALS

Urine from pregnant women.—Besides obtaining crystals from anterior-lobe tissue itself one would expect to find them in the urine of pregnant women since Aschheim and Zondek (1928) demonstrated the follicular stimulating hormone to be present there in large quantities. This was found to be true. Urine was made acid with acetic

and evaporated nearly to dryness. It was then extracted with ether to remove any oestrin present. An acid-alcohol extraction of the residue was made just as for anterior lobe, and typical crystals (Plate I, Fig. 2) were recovered, which, according to Table V, were equally as potent as anterior lobe. It is, however, quite impossible to have the same standardized dosage in both cases since crystals of anterior lobe were made up on the gram basis while those from urine were dissolved in enough water so that 1 cc. was equal to 126 cc. of unevaporated urine. Recent efforts seem to make it possible

TABLE V

Mouse No.	Dosage Daily	Started: Age in Days	Maturity: Age in Days	Remarks
	cc.			
I_{11}	0.1	17	21	
I_{12}	0.1	17	23	
Control				All killed at 34 days
I_{8}	0.1	17	20	
Control				All killed at 20 days
I_{69}	0.2	19	22	
Control				All killed at 25 days
I_{75}	0.05	18	21	
Control			40	
I_{78}	0.05	17	22	
Control			26	
Average	0.1	17.5	21.5	

to weigh the crystals and thus standardize the dosage from all sources in this manner—a far preferable procedure.

Epididymis of bull and fish sperm.—While preparing an extract of ground-up fresh bull epididymis for Professor Guyer for another purpose, it was found that, by using the same method of extraction as for the anterior lobe, crystals (Plate I, Fig. 3) were obtained which were identical in appearance to those from anterior lobe and from urine of pregnant women. These were injected as in Table VI into immature female mice and as can be seen precocious maturity resulted in the same average time as when the previous substances were used. In order to find out whether this hormone was a property of the germ cells themselves or other tissue, fish milt, which is prac-

tically a suspension of spermatozoa, was extracted and the crystals obtained were found to be equally active (Table VII). It would seem from these observations that probably the follicular stimulating hormone of the pituitary is present in many tissues of the body.

TABLE VI

Mouse No.	Dosage Daily	Started: Age in Days	Maturity: Age in Days	Remarks
	cc.			
M	0.25	20	22	
Control	.....	.....	.....	Both killed at 22 days
N	0.25	18	21	
Control	.....	.....	.....	Both killed at 21 days
I_2	0.15	14	21	
I_3	.15	14	21	
I_4	0.15	14	22	
Control	.....	.....	.....	All killed at 26 days
I_{33}	0.25	20	23	
I_{36}	0.25	18	23	
Control	.....	.....	31	
Average	0.207	16.9	21.8	

TABLE VII

Mouse No.	Dosage Daily	Started: Age in Days	Maturity: Age in Days	Remarks
	cc.			
I_{81}	0.1	18	20	
Control	.....	.....	.....	Both killed at 20 days
I_{81a}	0.1	18	20	
Average	0.1	18	20	

Adrenal cortex.—Besides the tissues already mentioned, desiccated adrenal cortex was extracted in the same manner. The cortical material yielded crystals of at least two different shapes. One of these was a typical anterior-lobe crystal while the other is quite different (Plate II, Fig. 4). When injected into immature mice precocious maturity resulted (Table VIII). Using the same technique, S. J. Martin

of this department obtained, from fresh beef anterior lobe and from thyroid-gland substance, crystals which were identical in appearance and in their physiological properties to those obtained from desiccated anterior lobe, epididymis, fish sperm, urine from pregnant women, and adrenal cortex.

Chemistry of anterior-lobe crystals.—Although the chemistry of the crystals of anterior lobe has not been determined to any extent, it

TABLE VIII

Mouse No.	Dosage Daily	Started: Age in Days	Maturity: Age in Days	Remarks
	cc.			
I_{112}	0.2	18	19	
I_{119}	0.2	18	19	
Control	.....	.....	.....	All killed at 19 days
I_{114}	0.2	18	23	
I_{116}	0.2	18	23	
Average	0.2	18	21	

TABLE IX

Mouse No.	Dosage Daily	Started: Age in Days	Maturity: Age in Days	Remarks
	cc.			
I_{101}	0.02	18	29	Crystals dried 3 weeks
I_{102}	.01	18	29	
I_{26}	.25	18	27	Extract made up in H_2O more than 2 weeks
I_{49}	0.25	18	26	
Average	0.132	18	27.7	

is probable that the active substance crystallizes from glacial acetic acid in combination with NaCl. Recrystallization from acetic acid always yields the same type of crystal. Drying in the crystalline form for any length of time injures the potency (Table IX); also the water solution loses its activity after a time (Table IX). Whether this is due to hydrolysis or bacterial destruction has not been determined. From the fact that crystals can be kept in glacial acetic acid indefinitely (six months at least) it is best to make the aqueous

extract from this as needed for injection. In solution the crystals are water clear and have a slightly salty taste. From such an aqueous solution the crystals cannot be recrystallized in the same characteristic shape.

THE EFFECTS OF OTHER FRACTIONS (EXCEPT CRYSTALS) FROM THE EXTRACTION OF ANTERIOR LOBE

It seems from all macroscopical or microscopical evidence that with the exception of the crystals (19*a*) and the luteinizing (25) fractions the effects from the residues resulting from acid-alcohol extraction of anterior lobe are entirely negative. Fractions (14*b*, 23*b*, 11*a*, 26*b*, Chart I) were injected into immature mice without any hastening of maturity, nor was the oestrus cycle of adult rats changed whatever.

Luteinizing fraction.—At step 21 all crystals can finally be separated from the brown material by dissolving the latter in absolute alcohol, the crystals not being soluble. When the alcohol is evaporated the residue is soluble in water. If this extract is injected in large quantities into rats it is quite toxic but does not seem to be so in smaller amounts. Soon after beginning the injections the animal goes into a state of dioestrus and remains so for long periods of time. The follicles apparently are not able to rupture but lutein cells form around the edge and grow toward the center inclosing the ovum. When injected into immature female mice no precocious maturity occurs. The vaginal membrane does not rupture earlier than normally, but soon after it does atretic corpora lutea are formed. Thus there is no evidence that atresia occurs before the follicles enlarge through normal maturity, but after they reach a certain size luteinization follows and ovulation is inhibited. It may be stated that the luteinizing reaction has been obtained up to the present time from anterior-lobe substance and urine of pregnant women only.

PRODUCTION OF PLACENTOMATA

Long and Evans (1922) in their study of the oestral cycle of the rat found that unless they could prolong the dioestral period they could not produce decidual growths by placing threads through the uterus. If, however, the cervix were stimulated, a period of pseudo-

pregnancy resulted and large placentomata could be induced if threads were placed through the uterus on the fourth day after oestrus and be left for 6 days until the animal was killed for examination. Placentoma formation, according to Evans and Simpson (1929*a*) is aided by injections of growth hormone, and by administering extracts of urine from pregnant women and from human placenta. Teel (1926) and Brouha (1928) have likewise observed that it is possible to obtain large placentomata by injecting anterior-lobe fluid.

The placentomata reaction then seemed to be another means of testing whether the follicular stimulating and luteinizing hormones were two distinct substances. A number of adult rats in oestrum were injected daily with crystals while others were given daily injections with the luteinizing fraction. After 4 days threads were placed in the uterus and the animals were injected for 6 more days at which time they were killed. It was found that every rat receiving the luteinizing hormone exhibited large decidual growths (Plate VI, Fig. 18), while in those receiving crystals no placentomata formed.

IS THE GROWTH HORMONE PRESENT IN EITHER THE CRYSTALLINE OR THE LUTEINIZING FRACTION?

This question cannot be answered definitely since long time injections of either substance have not been made. Teel and Watkins (1929) and Hewitt (1929) report that if growth hormone is present in alkaline extracts it can be detected in a short time by injecting adult rats. These rats gain quickly in weight over normal controls. This increase is not permanent if injections are stopped but serves as a good indicator for the presence of growth hormone. Teel's method was tried by injecting crystals and luteinizing hormone into adult rats for six weeks but the normal weekly fluctuations in weights were the same as the controls, with no increase in weight whatever (text Fig. 2).

DISCUSSION

Several investigators have reported at least partial separation of the hormones of the anterior lobe. Evans and Simpson (1928) obtain by alkaline extractions growth and luteinizing hormone, by acid extractions the sex-stimulating property. Hewitt (1929), by use of

filtered alkaline extracts of anterior lobe, observed rapid increase in weight of adult rats as well as a normal recurrence of oestrus in the females. If unfiltered extracts were injected, growth also increased but no oestrus was observed by the vaginal-smear method for a six-week period. While if anterior pituitaries were extracted with an acid aqueous solution, this extract caused precocious sexual maturity. He did not say whether or not injecting such material for a longer time would cause luteinization. By extracting beef anterior lobe

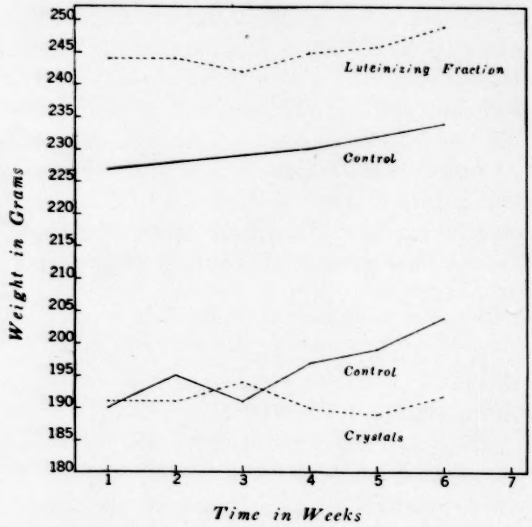


FIG. 2.—Growth-curves of adult rats injected with crystals and luteinizing fraction. Controls received no injection.

with mild alkali, Putnam (1928) obtained a growth-promoting hormone while the residue left after extraction with mild alkalies, acids, alcohol, and acetone was capable of producing premature oestrus in young rats and mice.

Wiesner (1930) prepared a sulphosalicylic acid extract of fresh human placenta which was oestrogenic, but which, if boiled, becomes kyogenic (causes mucification of the vagina). Boiling, according to this author, unmasks the luteinizing hormone. Also, by

treating placental substance with sulphosalicylic acid, Collip (1930) was able to obtain a very potent stimulating hormone of a microcrystalline nature. It would seem, then, that an alkaline extract yields growth hormone together with the luteinizing fraction, while from an acid extraction the follicular stimulating and the luteinizing fractions are obtained.

From the experimental evidence in this paper it would seem that an acid alcoholic extraction removes both the follicular stimulating and luteinizing principles and that these can then be separated by crystallizing the former from glacial acetic acid and removing the latter by means of its solubility in absolute alcohol.

Engle (1929) called attention to the fact that the action of transplants and urine from pregnant women upon the ovary differ. Ovulation follows treatment with transplants while luteinization and formation of atretic follicles follow injection of urine. Also, as observed by Evans and Simpson (1929*a*), the weight of the ovaries is proportional to the amount of tissue planted while the weight is scarcely increased after injecting urine of pregnant women.

Injections of crystals from anterior-lobe substance, as described in the present paper, are more like hypophyseal transplants in that precocious sexual maturity occurs and ovulation follows with no atresia, while the outcome is more like normal maturity in that superovulation does not occur. On the other hand, the luteinizing fraction causes atresia without ovulation.

Perhaps the most important problem which presents itself in the present study is that of whether this substance, obtained in the form of crystals or at least in association with a definite type of crystal, specific though it be, is a true hormone in the sense of a specific substance of relatively localized origin such as an endocrine gland. As has been pointed out, it not only has been obtained from anterior-lobe tissue, both fresh and desiccated, but also from the urine of pregnant women, the epididymis of the bull, trout sperm, adrenal cortex, and thyroid gland. The question of primary source and of possible later storage obviously arises here. Again, the possibility presents itself that hormone-like substances more general in nature and less local in origin than typical hormones, as commonly conceived, occur in living organisms, and that the substance in question

represents one of these. With these thoughts in mind further researches are being undertaken.

The non-crystalline luteinizing fraction, on the other hand, has been obtained only from the anterior-lobe substance and as a secondary derivative from the urine of pregnancy, and presumably, therefore, represents a hormone in the more restricted sense.

The writer wishes to express her appreciation to Professor M. F. Guyer and Professor F. L. Hisaw for their generous advice and interest during the progress of this study.

SUMMARY

1. A method of procedure with the anterior lobe of the pituitary body is described by which a substance producing precocious sexual maturity can be separated from one which causes luteinization of the follicles.

2. This substance, which apparently operates through follicular stimulation, has been obtained as a constituent of a crystalline fraction. The exact nature of the crystals is as yet undetermined. Whether, in fact, the substance is a true, specific hormone of the anterior lobe had perhaps at present best be left an open question (see par. 4).

3. These crystals when injected subcutaneously will induce precocious maturity in immature female mice and rats, and in immature male mice as well. They also stimulate follicular development in adult female rats.

4. The crystals in question have not only been obtained from anterior-lobe tissue, but likewise from the urine of pregnant women, the epididymis of the bull, fish sperm, thyroid gland, and adrenal cortex. Whatever their source, they manifest the same physiological properties. This widespread occurrence opens up the question of whether we may not be dealing with a substance more general in nature than a hormone as ordinarily conceived. This can only be answered through further experiment.

5. The only other active fraction obtained from the anterior pituitary substance by the procedure described was one soluble in absolute alcohol; it contained a luteinizing hormone. This hormone

was not obtained from similarly prepared extracts of adrenal cortex or of epididymis.

6. The luteinizing fraction causes, in the ovary of an adult rat, the formation of atretic corpora lutea with the ovum inclosed. This substance does not cause precocious maturity.

7. The crystals will not stimulate placentomata formation whereas the luteinizing hormone does.

8. After a six-week injection of the crystalline extract and the luteinizing fraction no increase in weight of adult rats was observable.

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PLATE I

FIG. 1.—Crystal from anterior lobe. ×262.

FIG. 1*a*.—Crystals from anterior lobe. ×65.

FIG. 2.—Crystals from urine of pregnant women. ×65.

FIG. 3.—Crystals from epididymis of the bull. ×65.

PLATE II

FIG. 4.—Crystals from adrenal cortex. ×65.

FIGS. 5, 6, and 7.—Ovary, uterus, and vagina of mouse I_2 matured at 21 days old with injections of crystals. ×65. Compared to the litter-mate control (Figs. 8, 9, 10) note the greater size, enlargement of follicles, and proliferation of cells of the vaginal mucosa.

PLATE III

FIGS. 8, 9, AND 10.—Ovary, uterus, and vagina of litter-mate control of I_2. Immature. ×65.

FIG. 11.—Ovary of rat 1 injected with crystals. ×19.

PLATE IV

FIG. 12.—Uterus of rat 1 injected with crystals. ×19.

FIG. 13.—Vagina of rat 1. ×65.

FIG. 14.—Ovary of rat 5 injected with luteinizing hormone. ×19.

FIG. 15.—Uterus of rat 5 injected with luteinizing hormone. ×19.

PLATE V

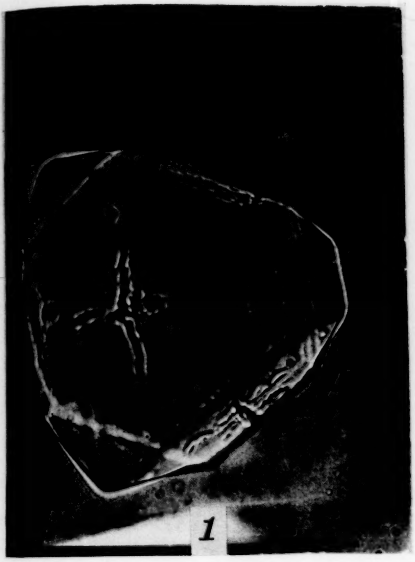
FIG. 16.—Vagina of rat 5 injected with luteinizing hormone. ×65.

FIG. 17.—Mouse E_3 injected with crystals, showing normal corpora lutea. ×65.

PLATE VI

FIG. 18.—The diagram on the left is the uterus of a rat injected with the luteinizing hormone showing a placentoma; on the right, one injected with crystals—no placentoma.

PLATE I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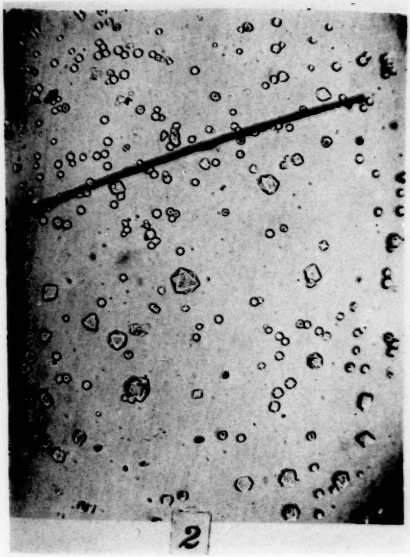
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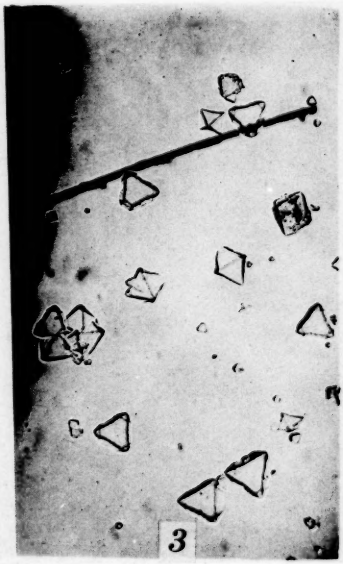
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PLATE II

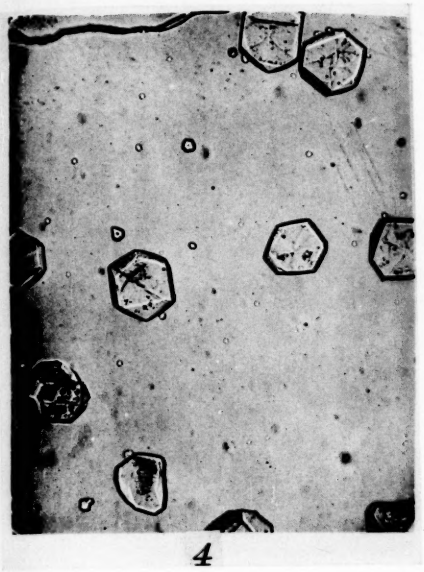

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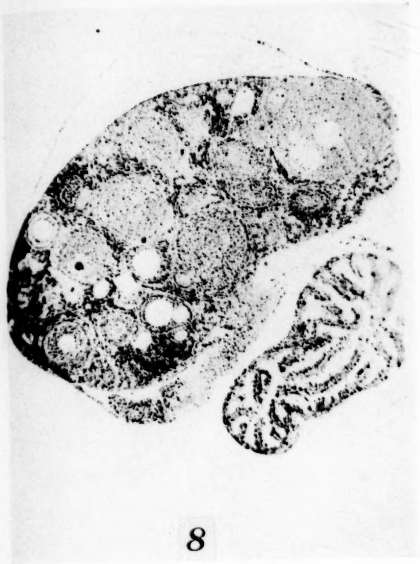
PLATE III



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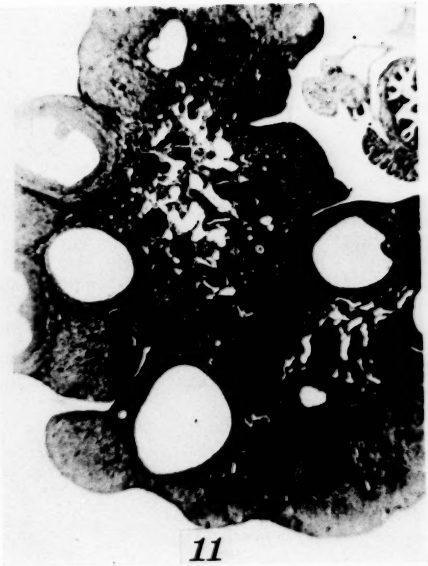

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PLATE IV

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PLATE V

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PLATE VI

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EFFECT OF SALTS, HYDROGEN-ION CONCENTRATION, AND PURE WATER ON LENGTH OF LIFE IN AMOEBA PROTEUS[1]

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INTRODUCTION

IT IS well-known that vital processes in cells and organisms are under various conditions profoundly affected by chemicals in the water surrounding them. All chemicals are toxic if the solution is sufficiently concentrated; but in lower concentrations some are under certain circumstances clearly beneficial. Nearly all marine organisms die very quickly in pure water and some vital processes in fresh-water forms do not occur in it. Root hairs, for example, do not develop unless the water surrounding the roots contains calcium (Hansteen-Cranner, 1914; Kisser, 1925; Farr, 1927). Indeed, it was formerly fairly generally held that pure water is toxic for all organisms. However, the results obtained by Nägeli (1893) on *Spirogyra*, by Daniel (1908) on *Paramecium*, and by Lynch (1919), Chambers and Reznikoff (1926), Mast (1928), and Dawson and Belkin (1929) on *Amoeba* show that all these organisms can live for several days in distilled water and that if chemicals aside from oxygen are necessary extremely low concentration suffices.[2] But they have no bearing on the question as to whether or not the length of life would have been increased by the addition of chemicals in proper

[1] I am greatly indebted to Mr. Keith Johnson for very efficient assistance, and to the Bache Fund for financial aid.

[2] In the observation made by Mast great precautions were taken to obtain pure water and to avoid contamination. The water used was redistilled in a tandem pyrex glass still described in a preceding paper (1928). The amoebae used were repeatedly washed in this water in carefully cleaned pyrex glass beakers, after which the water in the beakers was changed twice a day so as to avoid accumulation of waste products. In these experiments the content of the water, aside from CO_2 and O_2 received from the air, was therefore extremely low. In the other experiments mentioned the precautions taken to obtain and to maintain pure water were not so exacting, but in all of them the concentration of foreign substance in the water was doubtless low.

concentration. The results presented in the following pages concern this question, and they have a bearing on the optimum concentration and the relative toxicity of various substances.

MATERIAL AND METHODS

Amoeba proteus (Sch., 1916) raised in the laboratory and chemicals and water of the highest purity were used in all observations. The methods used in growing the amoebae and in purifying the chemicals and the water are described in a preceding paper (Mast, 1928).

In making the observations fifty to one hundred amoebae were taken from the culture in about 1 cc. of solution and put into a chemically clean pyrex glass beaker, containing 50 cc. pure water and left a few minutes, after which all that were in good condition were transferred with about 5 cc. solution to another clean pyrex glass beaker containing 50 cc. pure water. This was repeated five times, resulting in thorough washing of the animals and in the elimination of practically all the culture fluid carried over with them from the culture jar. During this process of washing nearly all of the specimens became radiate in form.

In making the tests 5 to 10 cc. (usually 10) of the solution under consideration were put into each of several 50-cc. clean pyrex glass beakers; 4 to 15 radiate specimens (usually 6) were then selected from those which had been washed and added to each beaker. Observations were then made on the time required for attachment and at frequent intervals thereafter on the number attached, the form, the activity, and the length of life.

The results obtained on form and attachment have been presented in preceding papers (Mast, 1928, 1929). Those obtained on length of life are presented in the following tables.

SINGLE SALTS

The results obtained in observations on the length of life in *Amoeba proteus* in pure water and in thirteen different salt solutions all N/1,000 are presented in Table I. These observations were all made during the same period, the amoebae used were all from the same culture and apparently of equal size and vigor, and the dishes in

which the observations were made were continuously subjected to the same environmental conditions.

By referring to this table it will be seen that the amoebae in pure water lived to a maximum of nearly 13 days. In this they attached to the substratum and moved normally and fission occurred showing that none of these processes is dependent upon substances in solution in the surrounding medium. It will be seen, however, that the length of life in some of the salt solutions was greater than in pure water, the maximum being more than 14 days for $CaCl_2$, NaCl, $NaNO_3$,

TABLE I*

EFFECT ON LENGTH OF LIFE OF VARIOUS SALTS, N/1,000

Salt Solution	Number Specimens	Time in Solution	Number Dead	Time in Solution	Number Dead	Time in Solution	Number Alive
$CaCl_2$	10	6 d	1	8 d	1	14 d	1
$Ca(NO_3)_2$	12	4	1	8	7	11	0
$CaSO_4$	6	7	1	8	1	10	0
$Ca_3(PO_4)_2$	9	5	1	8	3	11	0
$Ca(C_2H_3O_2)_2$	7	3	1	8	6	10	0
NaCl	10	5	1	8	4	14	3
$NaNO_3$	10	6	1	8	3	14	4
Na_2SO_4	9	8	1	8	1	14	3
Na_3PO_4	15	4	1	8	5	14	3
$NaC_2H_3O_2$	10	5	1	8	2	12	0
$MgCl_2$	10	7	1	8	1	14	3
KCl	9	5	2	8	8	9	0
NH_4Cl	12	2	1	8	11	9	0
H_2O	15	4	2	8	3	13	0

* Amoebae used in all tests taken from a distilled-water hay culture. d, day.

Na_2SO_4, Na_3PO_4, and $MgCl_2$ as compared with less than 13 days in pure water, indicating that all these salts in the concentration used protect the organisms. (Further evidence in reference to this will be presented later.) It will also be seen that on the whole the amoebae lived longer in the sodium salts than in any of the others tested with the possible exception of magnesium, and that the sodium salts are more favorable to longevity than the calcium salts. This is not only supported by the results of a comparison of the maximum length of life in these salts but also by the results of a comparison of the number dead on the eighth day, there being at this time 40.9 percent dead in the calcium salts and only 27.7 percent in the sodium salts. It will be seen, moreover, that in the potassium and the

ammonium salts the percentage dead at this time was much greater than in the other salts, but that in the magnesium salt it was less, being only 10 percent; however, not less than in several other individual salts.

The results presented in the table show that there was no marked difference in the effect of the anions tested with the exception of the acetate ions, which appear to be somewhat less favorable than the rest.

TABLE II*

EFFECT ON LENGTH OF LIFE IN *Amoeba proteus*, OF NaCl, KCl, AND $CaCl_2$ IN VARIOUS CONCENTRATIONS

Salt	Concentration	Time in Solution	Number Dead	Time in Solution	Number Dead	Time All Dead
NaCl.....	N/20	1 h	1	9 h	4	22 h
KCl.....	N/20	1 h	1	3 h	3	5 h
$CaCl_2$.....	N/20	8 h	1	.....	.....	?
NaCl.....	N/50	5 h	1	21 h	4	45 h
KCl.....	N/50	2 h	1	3 h	4	7 h
$CaCl_2$.....	N/50	45 h	0	7d	1	?
NaCl.....	N/100	21 h	1	.....	.....	?
KCl.....	N/100	3 h	3	.....	.....	7 h
$CaCl_2$.....	N/100	21 h	0	.....	.....	?
NaCl.....	N/500	48 h	1	7 d	1	?
KCl.....	N/500	8 h	1	22 h	3	2 d
$CaCl_2$.....	N/500	7 d	0	.....	.....	?
NaCl.....	N/1,000	7 d	0	.....	.....	?
KCl.....	N/1,000	1 d	0	2 d	2	6 d
$CaCl_2$.....	N/1,000	7 d	0	.....	.....	?
NaCl.....	N/5,000	7 d	1	.....	.....	?
KCl.....	N/5,000	7 d	0	.....	.....	?
$CaCl_2$.....	N/5,000	7 d	1	.....	.....	?

* *Amoebae* used in all tests taken from a culture consisting of modified Ringer solution and dialyzed timothy hay, pH 6.8. Six specimens used in each test. Temperature during tests 20° to 23°. h, hour; d, day.

The results as a whole show that in the salts tested the order of length of life, beginning with the highest, was somewhat as follows: sodium salts (SO_4, NO_3, Cl, PO_4, $C_2H_3O_2$); magnesium salts (?), pure water, calcium salts (Cl, SO_4, PO_4, NO_3, $C_2H_3O_2$); potassium salts, ammonium salts. This order does not, however, hold for all concentrations as the results presented in Table II show.

Table II shows that in concentration of N/20 the length of life is

relatively low for the three salts tested, but that it is clearly lowest for KCl and highest for $CaCl_2$, indicating that of the three cations tested the potassium ions are most toxic and the calcium ions least. This is in accord with results obtained by Chambers and Reznikoff (1926). The table also shows for all three salts tested that as the concentration decreases the length of life increases to a maximum and then apparently remains constant, but that the concentration in which the maximum is reached differs greatly, it being between N/100 and N/500 for $CaCl_2$, N/500 and N/1,000 for NaCl, and N/1,000 and N/5,000 for KCl. It indicates that in N/5,000 the effect of the three salts is the same. Similar results were also obtained in tests made with still lower concentrations. Observations were also made with pure water, but they were not continued long enough to ascertain the relation between the length of life in this and in salts in optimal concentration.

The results presented in Tables I and II appear to show that sodium salts are more toxic than calcium salts in strong solutions and less toxic in weak ones; i.e., they indicate that relative toxicity of salts depends upon the concentration.

Chambers and Reznikoff (1926) made some interesting experiments on *Amoeba*, and they concluded that the toxicity of salts depends upon whether they act on the surface or on the internal substance. They say (p. 399), "NaCl and KCl are more toxic to the exterior of the cell [*Amoeba proteus*] than to the interior, and the reverse is true for $CaCl_2$ and $MgCl_2$." This conclusion is based upon a comparison of the effect produced by injecting into amoebae salt solutions of given concentrations with the effect produced by immersing amoebae in salt solutions of the same concentrations. They found that injection produces greater injury than immersion if NaCl or KCl is used, but less if $CaCl_2$ or $MgCl_2$ is used. These results appear to be well established, but the conclusion reached is decidedly equivocal; for the results obtained under the two conditions which obtained in the tests are not comparable. This is clearly demonstrated by the following considerations:

If a droplet of salt solution of a given concentration is injected into an amoeba, it is localized and it immediately, owing to diffusion, becomes greatly diluted and consequently acts in the original concentration only momentarily; while if an amoeba is immersed in a

salt solution of a given concentration the entire surface is in contact with the solution, i.e., a much larger surface than in case of injection, and the action continues indefinitely with practically no change in concentration. Moreover, it is generally held that NaCl and KCl enter cells freely. If this obtains it is altogether likely that if an amoeba is immersed in a solution of either of these salts of a given concentration, the concentration in the interior of the amoeba soon becomes greater than it would be if a droplet of either of these salts of the same concentration had been injected. If this obtains one would expect greater injury from immersion than from injection, not, however, by action on the surface in case of immersion and on the interior in case of injection, as maintained by Chambers and Reznikoff, but by action on the interior under both conditions. $CaCl_2$ and $MgCl_2$, on the contrary, enter cells very slowly. The concentration of these salts in the cytoplasm in amoebae immersed in solutions of them will, therefore, in all probability, increase much more slowly than it will if solutions of these salts of the same concentration are injected. If this is true, one would expect injection to produce greater injury than immersion, i.e., the reverse from what would be expected with NaCl or KCl.

If all this is true, the results obtained by Chambers and Reznikoff obviously do not demonstrate that NaCl and KCl are more toxic if they are in contact with the surface of an amoeba than they are if they are in contact with the cytoplasm in it; and they obviously do not demonstrate that the injurious action of NaCl or KCl differs radically from that of $CaCl_2$ or $MgCl_2$.

Table III indicates that the effect of sulphates is essentially the same as the effect of chlorides (cf. particularly the results obtained in K_2SO_4 and Na_2SO_4, N/1,000, with those obtained in NaCl, N/1,000), but that the carbonates are much more toxic. This, however, is doubtless owing to difference in the hydrogen-ion concentration, the carbonates being very strongly alkaline in the higher concentrations and apparently not toxic in concentrations in which the hydrogen-ion concentration is favorable, e.g., N/10,000 in which it is pH 6.8. These results therefore support the conclusion reached in reference to the anions in Table I; namely, that the action on *Amoeba proteus* of the different anions is essentially the same.

By comparing the results obtained in the most favorable salt solutions recorded in the table (Na_2SO_4, N/1,000; NaCl, N/1,000;

K_2SO_4, N/100,000) with those obtained in pure water, it will be seen that the maximal length of life in pure water was practically the same as it was in these salt solutions. This, however, did not regularly obtain in other experiments. By referring to Tables I, III, IV and VII it will be seen that seven tests were made in which the length of life in pure water can be directly compared with that in salt solutions and that in five of these tests (Tables I, III, IV, VII) it was clearly greater in the salt solution than in pure water, while

TABLE III*

EFFECT OF SULPHATES AND CARBONATES ON LENGTH OF LIFE IN *Amoeba proteus*

	Length of Life in Days											
	N/100		N/1,000		N/10,000		N/100,000		NaCl N/1,000		H_2O	
	Aver.	Max.	Aver.	Max.	Aver.	Max.	Aver.	Max.	Aver.	Max.	Aver.	Max.
Na_2SO_4	1⅜	2	11⅔	15	?	?	6⅓	12	11⅘	15	....	....
K_2SO_4	1—	1—	10⅚	12	8⅗	12	11½	16	....	....	....	....
Na_2CO_3	2 min.	5 min.	6	10	8½	11	10⅖	12	11⅓	18	11⅕	16
K_2CO_3	1½ min.	4 min.	1—	1	8	11	7	10	10⅗	16	11⅖	14
Total average	.....	.....	....	....	....	....	....	....	11 4/5	16⅓	11 1/25	15

* Amoebae in all tests taken from an old culture containing blue-green algae, pH 7.2 to 8.9, depending upon time of day. Six specimens used in each test. Temperature during tests 20° to 23°. Hydrogen-ion concentration Na_2SO_4, K_2SO_4, NaCl, and H_2O, pH 6.2; Na_2CO_3 and K_2CO_3, N/100 and N/1,000, pH 9+; N/10,000, pH 6.8; N/100,000, pH 6.4.

in two (Tables III, IV) it was practically equal, indicating that salt in proper concentration (N/1,000±) tends to prolong life. This is strongly supported by the averages of the results obtained in the seven tests mentioned, in which it is found that the total average length of life in pure water was 8⅓ days and in salt solution 9⅔ days, and the average maximum in pure water 11⅔ days and in salt solution 13⅘ days. It is also supported by results obtained with balanced salt solutions.

BALANCED SALTS

The more important results obtained with balanced salt solutions are presented in Table IV. This table contains the results obtained in two experiments. They show that the length of life in optimal salt concentration was, in both experiments, much greater than in water, the difference being markedly greater than that observed in

any of the experiments with single salts, indicating that the protective action of balanced salts is greater than that of single salts. This is also indicated by the fact that in one of the experiments the length of life in the balanced salt solution at optimal concentration was considerably greater than it was in NaCl solution.

How it is that salt protects the amoebae is not definitely known. It might be suggested that it causes a reduction in activity and metabolism in general, but the evidence presented in a preceding paper

TABLE IV*

EFFECT OF CONCENTRATION OF SALTS IN BALANCED SOLUTION, CONSISTING OF $CaCl_2+KCl+NaOH+NaH_2PO_4$, ON LENGTH OF LIFE IN *Amoeba proteus*

CONCENTRATION		LENGTH OF LIFE IN DAYS			
		Exper. 24		Exper. 27	
Na	K and Ca each	Aver.	Max.	Aver.	Max.
N/10	N/200	1—	1—	1—	1—
N/25	N/500	1½	2	1—	1
N/50	N/1,000	9	11	4⅘	7
N/500	N/10,000	9⅗	11	10⅓	20
N/2,500	N/50,000	6⅔	9	14⅗	19
N/5,000	N/100,000	5⅔	6	10⅗	22
N/10,000	N/200,000	4⅔	7	4	7
NaCl N/1,000		9⅗	11	9⅗	12
H_2O		5	6	9⅗	12

* Hydrogen-ion concentration of balanced salt solution, pH 6.6; of NaCl and of H_2O, pH 6.2.

Amoebae used in experiment 24 taken from an old spring-water, dialyzed hay culture, pH 7.2; in experiment 27 from a new spring-water, dialyzed hay culture, pH 6.6. Five specimens used in each test. Fission occurred in experiment 27, N/50,000. Temperature during tests 20° to 23°.

(1929) shows clearly that amoebae in weak solutions attach more readily and firmly and are in general more active than in pure water, indicating that metabolism is higher in the former than in the latter. This, in animals without food, would obviously tend to decrease the length of life in the salt solution in comparison with that in pure water.

It has been demonstrated (1929) that the surface of amoebae in weak salt solution is much more adhesive than it is in pure water, and it may well be that this difference is associated with difference in permeability, of such a nature that there results a difference in the loss of substances that are of vital importance—vitamins and

salts, for instance—and that this accounts for the difference in length of life observed.

It is, however, not surprising that amoebae live longer in salt solution than in pure water. The surprising thing is that they live as well as they do in pure water. The fact that they do shows that they have a highly developed regulatory mechanism which prevents excessive loss of substances in solution in the numerous vacuoles found scattered all through the cytoplasm (see Mast, 1926).

By comparing Table IV with Table I it will be seen that the results indicate that in the stronger solutions used the single salts are relatively much more toxic than the mixed salts. These tables show, for example, that the average length of life in NaCl N/50 was only about 1 day, while in NaCl N/50+KCl and $CaCl_2$ it was 9 days in one experiment and $4\frac{4}{5}$ days in the other. This is in harmony with results obtained by Reznikoff (1928) on *Amoeba dubia* and many others on other organisms.

Table IV also shows that the optimal concentration of the salts and the length of life in it depend upon the condition of the culture from which the amoebae are taken. The amoebae used in experiments 24 and 27, respectively, were taken from cultures which differed radically. Those used in the former were taken from an old culture which was slightly alkaline (pH 7.2); those used in the latter from a new culture which was acid (pH 6.6). In the one the optimum concentration was considerably higher and the length of life considerably less than in the other.

HYDROGEN-ION CONCENTRATION

Chambers and Reznikoff (1926) maintain that hydrogen concentration of pH 5.6 is fatal to *Amoeba proteus* in less than 1 day, but that increasing the alkalinity from pH 7 to 9.8 does not alter the length of life. The results presented in Tables V–VII indicate much lower toxicity than this in the acid range and much higher in the alkaline range, and a definite optimum under certain conditions.

Table V shows in all three experiments represented that there was a fairly definite optimum, that in two of the experiments the optimum was around pH 6.6–7, probably a little to the acid side of neutrality, and in the other probably practically at neutrality. This

difference in the location of the optimum may have been due to the difference in the cultures from which the amoebae were taken, those used in experiments 8 and 9 having been taken from an old culture (pH 7.2–8.9) and those used in experiment 10 from a new culture (pH 6.8). The data at hand are, however, so meager that definite conclusions cannot be drawn concerning this matter.

By comparing Tables V and VI it is at once evident that the optimum in both experiments represented in the latter was considerably more acid than it was in any of the experiments represented in

TABLE V*

EFFECT OF HYDROGEN-ION CONCENTRATION ON LENGTH OF LIFE IN *Amoeba proteus* IN KOH M/1,000+KH_2PO_4 M/1,000 IN VARIOUS PROPORTIONS; K=N/1,000 IN ALL

pH AT BEGINNING OF EXPER.	LENGTH OF LIFE IN DAYS						pH at Close of Exper.
	Exper. 8		Exper. 9		Exper. 10		
	Aver.	Max.	Aver.	Max.	Aver.	Max.	
5.5	$4\frac{1}{6}$	5	$4\frac{1}{2}$	8	$1\frac{3}{4}$	2	5.5
6.2	$6\frac{5}{6}$	9	6	9	$2\frac{1}{2}$	3	6.2
6.6	$8\frac{1}{3}$	9	$7\frac{5}{6}$	11	2	4	6.6
7.0	$7\frac{2}{3}$	11	$6\frac{1}{6}$	11	$4\frac{1}{3}$	6	6.8
7.4	$5\frac{5}{6}$	8	4	9	$1\frac{1}{2}$	3	7.2
7.8	$4\frac{1}{3}$	8	$7\frac{4}{5}$	9	1—	3	7.4

* Amoebae used in experiments 8 and 9 taken from an old culture containing blue-green algae, pH 7.2–8.9, depending upon time of day; those used in experiment 10 from a new culture of unknown composition, pH 6.8. Six specimens used in each test. Those used in experiment 10 treated with weak solution of neutral red.

the former, it being at pH 5± in the one and at 7± in the other. This is in all probability owing to the difference in the concentration of the salt in the two sets of experiments, it being N/1,000 in the one and N/100 in the other. This indicates that the effect of hydrogen-ion concentration on *Amoeba* is specifically correlated with the concentration of salts and probably also with other substances in the solution. This is in harmony with results obtained by Farr (1928, p. 17) on the growth of root hairs. Farr contends, however, that the shift of the optimum is due to insufficient calcium to antagonize the hydrogen ions in the weaker solutions, i.e., he contends that there is antagonism between acid and calcium. This cannot hold for *Amoeba*, because the shift was observed in a solution that contained no cal-

cium, unless it is assumed that it is the calcium within the organism that protects it, and that in the weaker solution this leaches out, resulting in loss of protection.

Chambers and Reznikoff (1926) maintain that in their actions on *Amoeba* there is antagonism between NaCl and HCl. Our observations indicate that this is not specific for NaCl.

The results presented in Table VII show that *Amoeba proteus* lives fairly well over a surprisingly wide range of hydrogen-ion concentrations. They show that in the experiments represented some

TABLE VI*

EFFECT OF HYDROGEN-ION CONCENTRATION ON LENGTH OF LIFE IN *Amoeba proteus* IN NaOH M/100+NaH_2PO_4 M/100 IN VARIOUS PROPORTIONS; Na=N/100 IN ALL

pH AT BEGINNING OF EXPERIMENT	NUMBER OF SPECIMENS LIVED TWO DAYS		pH AT CLOSE OF EXPER.
	Exper. 17	Exper. 18	
4.6	all	all	5.2
5.2	all	all	6.2
6.6	2	4	6.6
7.0	2	0	7.0
7.4	0	0	7.4
7.8	0	0	7.8
NaCl N/1,000 6.2	all	all	6.2
H_2O 6.2	all	all	6.2

* Amoebae used in experiment 17 taken from a culture of unknown composition (pH 7.0). Those used in experiment 18 from a spring-water, dialyzed hay culture (pH 6.8). Five specimens used in each test. Fission occurred in experiment 18, pH 4.6, and in NaCl.

specimens lived 8 days in hydrogen-ion concentration of pH 3.8–4.2 and others 7 days in a concentration of pH 8.2–7.8, and that in these experiments there was no marked difference in the length of life over the entire range between these concentrations, although there appears to have been a slight increase around pH 6.6 or 7 and another around pH 5. There is, in fact, in all of the experiments some indication of a double optimum located in these regions, i.e., pH 6.6–7 and pH 4.6–5.3. Hopkins (1928) concluded on the basis of the relation between hydrogen-ion concentration and the rate of locomotion in *Amoeba* that there is a double optimum concentration with a median minimum at pH 7. My results are consequently in accord with

Hopkins in so far as they indicate a double optimum, but they are not in accord with his in reference to the location of the optima. He contends that the median minimum is near pH 7. My results indicate that it is near pH 6. This difference may, however, be due to difference in the salt content of the solutions used in the two sets of experiments.

TABLE VII*

EFFECT OF HYDROGEN-ION CONCENTRATION ON LENGTH OF LIFE IN *Amoeba proteus* IN BALANCED SALT SOLUTION CONTAINING $CaCl_2+KCl+NaOH+NaH_2PO_4$; NaOH M/1,000 AND NaH_2PO_4 M/1,000 IN VARIOUS PROPORTIONS; CONCENTRATION IN ALL SOLUTIONS; Ca N/1,000, K N/1,000, Na N/200

pH AT		LENGTH OF LIFE IN DAYS							
		Exper. 19		Exper. 21		Exper. 22		Exper. 23	
BEGINNING	CLOSE	Aver.	Max.	Aver.	Max.	Aver.	Max.	Aver.	Max.
3.2	?	1—	1—	1—	1	.....	.....	1—	1
3.4	3.8	.....	.....	$1\frac{4}{5}$	2	$1\frac{1}{2}$	4	.....	.....
3.8	4.2	.....	.....	5	8	.....	.....	.....	.....
4.2	4.9	.....	.....	$6\frac{1}{5}$	9	$6\frac{3}{5}$	8	$6\frac{1}{5}$	10
4.6	5.0	$7\frac{1}{2}$	9	$6\frac{2}{5}$	10	5	8	$5\frac{2}{5}$	11
5.0	5.3	.....	.....	10	14	$5\frac{3}{5}$	7	$4\frac{1}{5}$	5
5.4	5.6	.....	.....	$7\frac{3}{5}$	12	.....	.....	.....	.....
5.8	6.0	.....	.....	$6\frac{2}{5}$	11	$4\frac{2}{5}$	6	$5\frac{3}{4}$	8
6.2	6.2	10	12	.....	.....	.....	.....	.....	.....
6.6	6.6	$10\frac{1}{2}$	15	.....	.....	$3\frac{1}{2}$	6	4	4
7.0	7.0	$9\frac{1}{6}$	15	.....	.....	$6\frac{2}{3}$	13	$5\frac{1}{4}$	7
7.4	7.3	$6\frac{2}{3}$	14	.....	.....	$3\frac{2}{5}$	5	$3\frac{1}{2}$	4
7.8	7.6	$8\frac{5}{3}$	14	.....	.....	.....	.....	.....	.....
8.2	7.8	.....	.....	.....	.....	$3\frac{4}{5}$	5	5	7
8.8	8.0	1	2	.....	.....	$1\frac{1}{5}$	2	1—	1
NaCl N/1,000	6.2 ...	$10\frac{2}{3}$	14	11	16	.....	.....	7	10
H_2O N/1,000	6.2 ...	$8\frac{5}{6}$	12	.....	.....	.....	9	$4\frac{4}{5}$	6

* Amoebae used in experiments 19 and 21 taken from a spring-water, dialyzed hay culture, pH 6.8; those used in experiment 22 from a new spring-water, dialyzed hay culture, pH 6.7, and those used in experiment 23 from a modified Ringer solution, hay culture, pH 7.7.

Five specimens used in each test. Fission occurred in experiment 19, pH 6.2.

SUMMARY

1. *Amoeba proteus*, without food, in water of very high purity moves normally, divides, and lives several days; a maximum of 16 days was observed.

2. Salt in proper concentration, both single and mixed, prolongs the life; a maximum of 18 days in the former and 22 days in the latter was observed.

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3. In single salt solutions N/1,000 the order of length of life observed, beginning with the highest, was as follows: sodium salts (SO_4, NO_3, Cl, PO_4, $C_2H_3O_2$), magnesium salts (?), calcium salts (Cl, SO_4, PO_4, NO_3, $C_2H_3O_2$), potassium salts, ammonium salts. There was only a very slight difference in the effect of the different anions used excepting $C_2H_3O_2$, which was considerably more toxic than the rest.

4. The relative toxicity of different salts depends upon the concentration; for example, Na is more toxic than Ca in concentrations from N/20 to N/500 but less toxic in a concentration of N/1,000.

5. The conclusion reached by Chambers and Reznikoff (that "NaCl and KCl are more toxic to the exterior of the cell than to the interior and [that] the reverse is true for $CaCl_2$ and $MgCl_2$") is equivocal.

6. Carbonates in strong solutions are much more toxic than sulphates and the other salts tested, but this is doubtless owing to difference in hydrogen-ion concentration, the carbonates being strongly alkaline.

7. The length of life in strong solutions is much lower in NaCl alone than in NaCl of the same concentration with KCl and $CaCl_2$ added.

8. *Amoeba proteus* can live in a surprisingly wide range of hydrogen-ion concentrations. Some specimens lived 8 days in pH 3.8–4.2 and others 7 days in pH 8.2–7.8. In concentrations between these there is no marked difference in the length of life, but there is some indication of a double optimum, one at pH 6.6 or 7 and another at pH 6.4 or 5. Beyond pH 4.2 and 8.2 the length of life decreases very rapidly.

9. This holds for a solution containing Ca N/1,000+K N/1,000 +Na N/200. In stronger concentration the optima doubtless are more acid. In general, the effect of hydrogen-ion concentration depends upon the salt concentration. The stronger the solution, the more acid the optimum, i.e., the less the effect of the hydrogen ions.

10. The effect of salts and hydrogen ions depends upon the condition of the culture from which the amoebae are taken.

11. Fission is not specifically dependent upon the composition of the solution in which the amoebae are, provided it is not too con-

centrated. It was observed in the following solutions: pure water, K_2SO_4 N/1,000; Na_2SO_4 N/100,000; NaCl N/1,000; NaOH+NaH_2PO_4, pH 4.5; $CaCl_2$+KCl+NaOH+NaH_2PO_4, pH 6.2; and in a number of other single salts, N/1,000.

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AVAILABLE BACTERIA AND THE SEX RATIO IN MOINA

(Three figures)

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PARTHENOGENETIC *Moina* reared in a favorable environment almost invariably produce only female young, but when experimentally crowded give rise to many males. This statement is amply supported by the earlier contributions of Grosvenor and Smith (1913) and later ones of Banta and Brown (1929*a*, *b*, *c*, *d*, *e* and 1930). The latter, working especially with *Moina macrocopa*, found that in general approximately 40 per cent males were produced when ten or twelve mothers were crowded in 75 cc. or ml.[1] of culture medium, while uncrowded controls released only female young. From numerous experiments it was inferred that the accumulation of excretory products was the principal factor in stimulating male production by crowded mothers. Since each of the ten mothers crowded in 75 ml. of medium frequently produced as large a brood as a single mother in the same amount of medium, it was suggested (Banta and Brown, 1929*a*) that the amount of available food in the medium was but slightly related to the production of males.

More recent investigations, however, in which the amount of food available to crowded mothers was carefully controlled, do not substantiate this suggestion. A previous report by Stuart and Banta (1929) indicates that quantity of food may under certain conditions appear to be the major influence in the control of sex in *Moina macrocopa*. The purpose of the present paper is to expand the work previously reported by presenting additional experimental data, and to describe more fully the procedure employed in the experiments. Acknowledgment is gladly made to Maurita E. McPherson for efficient and exceptionally faithful help in carrying out the experiments

[1] Ml. = milliliters.

reported in this paper and to the Department of Genetics of the Carnegie Institution of Washington for making it possible for her to aid in this work.

METHODS AND MATERIALS

The problem of varying the amount of available food was first approached by crowding mothers in different concentrations of the medium described by Banta (1921). This procedure was very unsatisfactory as in the higher concentrations of the manure infusion the Cladocera often died in great numbers, or should they survive to release their broods, the counting and sexing of the young became an extremely laborious task accompanied by the danger of errors because of the dense color and turbidity of the medium.

The fact that the concentrated medium was toxic for the Cladocera while sufficient dilution rendered it non-toxic suggested that a medium, even if not toxic for the bacteria present, might at least inhibit their growth and that sufficient dilution of the medium could have a marked influence on bacterial reproduction. To test this assumption eleven different batches of manure infusions prepared at varying intervals during one summer were tested in the following manner. The freshly strained concentrated infusion was diluted 2.5, 5, 7.5, 10, 15, and 25 times in wide-neck 200-ml. bottles with pond water, and together with a similar bottle of the concentrated infusion were incubated at 26°–28° C. Bacterial counts were made from each batch of the infusion as soon as it was diluted; a second count was also made 24 hours later on some of the batches; 36 hours later on some, and 48 on others. Any influence of dilution of the medium on the numbers of bacteria present in the original infusion can easily be seen in a comparison of the first and second counts. The several counts obtained at the time the infusion was diluted were averaged together, as were all the counts made after incubating 24, 36, or 48 hours, and plotted in Figure 1.

These curves show very clearly that up to a certain point mere amount of dilution of a concentrated medium with pond water provides no safe criterion of a decrease in the bacterial count proportional to the dilution. Thus a medium containing 20,000,000 bacteria per milliliter diluted 10 times with pond water will have approximately 2,000,000 per milliliter immediately after dilution, but 24

or more hours later it may have as high as 15,000,000 bacteria per milliliter. The pond water used in diluting the medium may have introduced new species of bacteria or more food for bacteria, or diluted substances already present, which are inimical to the growth of the bacteria. All three factors may presumably be active here (Graham-Smith, 1920; Speakman and Phillips, 1924; Schmidt and

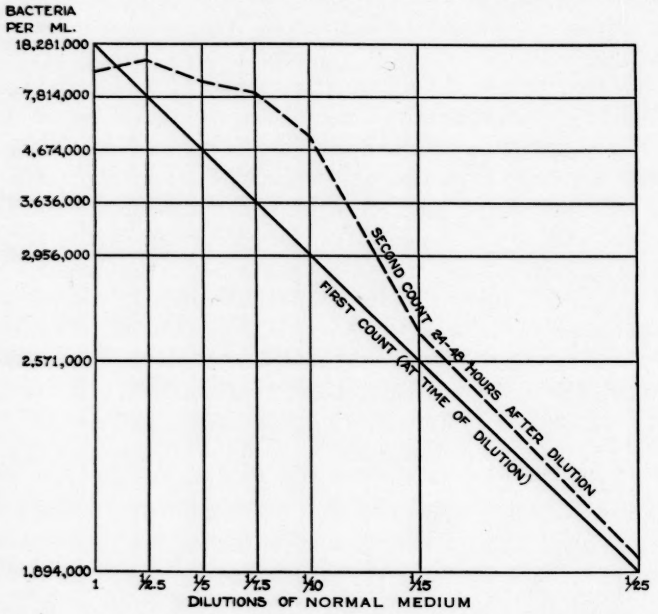

FIG. 1.—Numbers of bacteria in manure solution culture medium subsequent to dilution. Scale of axis of ordinates so arranged that the curve of bacterial numbers immediately after dilution (first counts) forms a straight line. Second counts made 24 to 48 hours after dilution. The data employed are averages of eleven such determinations.

Griefenstein, 1924). Only when a medium has been diluted 15–25 times with pond water will the bacterial count be proportional to the dilution even after 48 hours.

Considerable variation was found in the numbers of bacteria not only in the different makeups of the medium, but also in the different lots of pond water or in the same lot of pond water after it had re-

mained in the laboratory for some time. The highest count obtained with any batch of medium before dilution was 28,000,000 and the lowest 1,500,000 bacteria per milliliter. The highest count for pond water equaled 3,000,000 and the lowest less than 100,000 per milliliter. The effect of dilution on the bacterial population of a medium also varied with the different batches of infusions. In some cases after moderate dilution (2.5–5 times) the second count exceeded that obtained for the concentrated medium, while in others the count following dilution and incubation was nearly proportional to the dilution. Such variations may be due to the presence of certain substances in the manure, soil, or even pond water which exert a marked influence on bacterial growth.

Such variation and complications preclude the use of varying dilutions of the usual manure infusion in experiments on the control of sex with Cladocera by means of variation in the amount of available food present, since such experiments necessitate the use of a medium to which bacteria can be added without there being any great increase or decrease in their numbers over a period of 48 hours. The sex of the forthcoming young will be determined by this time (mothers 48 hours old) if the mothers are maintained at a temperature of 26° C., so that any amount of fluctuation in the bacterial count after this period is not significant (in so far as the sex of the first clutch of young is concerned). With this in mind an attempt was made to cultivate *Moina macrocopa* mothers in autoclaved pond water to which had been added varying amounts of pure cultures of several species of bacteria. Cladocera were reared in this manner for several generations, but were not considered satisfactory for experimental purposes as the mortality was high and the clutches of young were small. The use of pond water that had been sterilized by Berkefeld filtration was even less successful. After many attempts to secure a suitable medium the following was devised:

Concentrated manure infusion was diluted 5–9 times, depending on its original density, with pond water; silt was added in the usual manner since it was found to aid in the flocculation of suspended material. This mixture was allowed to stand at room temperature for 4–5 days with occasional stirring. It was then filtered through hard filter paper, the first part of the filtrate being returned to the

paper to insure clarity of the medium. Bacteria added to this filtrate in the course of 48 hours usually show a slight decrease in numbers though occasionally a marked increase may occur in some filtrates.

In a series of 30-ml. bottles containing 10 ml. of the filtrate were placed increasing amounts of *Aerobacter aerogenes* which had been cultivated on agar slants. The growth was washed from a slant with 6 ml. of the filtrate and to the first bottle 0.1 ml. of the bacterial suspension was added, to the second 0.2 ml., to the third 0.3, etc., up to 1 ml. Best results seemed to have been obtained with cultures of the bacteria that had been cultivated at room temperature for at least 48 hours. A single *Moina macrocopa* mother was placed in each bottle and the growth and releases compared with the control, a sister-mother reared in normal medium (the usual dilution of manure solution). In the bottles containing 0.1–0.5 ml. of the bacterial suspension growth was somewhat retarded and the releases smaller than in the controls. Bottles having from 0.6 to 1.0 ml. of the suspension gave results equal to or better than the control bottles. From this it would seem that a medium had been secured in which the bacterial number could be varied at will, and which in turn could be made to control the growth of Cladocera, retarding or accelerating the growth as desired.

Preliminary experiments in the control of sex by the amount of available food present were made by crowding from three to five mothers in 9.25 ml. of the filtrate to which had been added 0.75 ml. of the bacterial suspension. Controls were run with the same number of mothers in 10 ml. of normal medium. In every case experimental and control bottles contained sister-mothers. As soon as the mothers released, the sex and the total number of young in each bottle were determined. The results tabulated in Table I were obtained from twenty-six experiments employing seven different lots of bacterized filtrates and several different generations of cladoceran mothers.

Mothers crowded in the normal medium produced a total of 1,364 young of which 49.27 per cent or 672 were males, while the mothers crowded in the bacterized filtrate produced 1,542 female young and not a single male. Moreover, the total number of young released in the filtrate was 13.1 per cent greater than in the normal medium.

From seventy similar experiments (to be reported later in this paper) it was found that in a filtrate containing the most favorable number of bacteria crowded mothers will produce approximately 20 per cent more young than sister-mothers in the same quantity of the normal medium. This fact alone would tend to show that a properly bacterized filtrate is a more favorable medium than the normal infusion in that the latter perhaps lacks an adequate number of bacteria.

Assuming that an adequate amount of bacterial protein in the filtrate might be the controlling factor in producing only females in

TABLE I

Series No.	No. of Bottles	No. of Mothers per Bottle	Normal Medium		Bacterized Filtrate	
			Total Young	Per Cent ♂'s	Total Young	Per Cent ♂'s
1	3	5	125	32	209	0
2	4	4	177	59.8	201	0
3	4	3	260	65.7	269	0
4	3	3	171	46.7	194	0
5	4	3	230	39.1	253	0
6	4	3	219	49.3	231	0
7	4	3	182	37.9	185	0
Totals and average Per cent ♂'s	26	88	1,364	49.27	1,542	0

the bacterized filtrate used in the preceding experiments, experiments were conducted in which increasing quantities of bacteria were added to successive bottles of this filtrate in the hope that a sex gradient, dependent upon the number of bacteria present per bottle, might thereby be produced. Accordingly, 50, 40, 45, 46.7, 48, 49, and 49.5 ml. of the filtrate were placed in 120-ml. wide-neck bottles. The growth from 10 agar-slant cultures of *Aerobacter aerogenes* (reared for about 48 hours at approximately 26° C.) was suspended in about 30 ml. of the filtrate and added to the bottle containing 50 ml. of the filtrate. This was called dilution 1. After thorough mixing, 10 ml. of this dilution were transferred to the bottle containing 40 ml. of filtrate, thus resulting in a 1:5 dilution of the bacterial suspension referred to above as dilution 1. Again 5 ml. of dilution 1 were placed in the bottle containing 45 ml. of the filtrate giving a 1:10

dilution. This was repeated until dilutions 1, 1:5, 1:10, 1:15, 1:25, 1:50, and 1:100 had been secured. After thoroughly mixing each dilution, 10-ml. amounts of the different dilutions were pipetted into as many 15-ml. wide-neck bottles as desired. It was found impracticable to place the required amount of filtrate in the small bottles and to add the necessary amount of concentrated bacterial suspension with a serological pipette, because the suspension might contain as many as 4,000,000,000 bacteria per milliliter and a discrepancy of .01 ml. in pipetting would introduce an error of 40,000,000 bacteria in a single bottle.

Three young female *Moina macrocopa* were placed in each bottle of a series, so distributed that all the experimental animals in any one series were sisters. In the later experiments two more bottles were added to each series, one containing 10 ml. of unbacterized filtrate and three mothers, the other 10 ml. of normal medium and three mothers. In all experiments at least two series of bottles were used in order that the reaction of different clutches of *Moina* could be studied in identical environments. In one experiment as many as eight clutches were used. As the experimental animals in the various bottles released their first clutches of young the approximate time of each release was noted and the total number and sex of the young were determined. Seventy such series were studied. The results of twenty-two of these series are tabulated in Table II and shown in Figure 2.

It will be seen from Table II and Figure 2 that the control of the sex of the young produced by crowded *Moina macrocopa* mothers can be easily accomplished by varying the numbers of bacteria as available food in the medium. Two hundred and ten mothers crowded in the concentrated bacterial suspension (col. 3 in Table II) did not produce a single male, while the same number of mothers in a 1:50 dilution of the concentrated suspension produced 60.1 per cent males. Sex control in these experiments is by no means perfect since there is considerable variation in the different series. In several cases, however, a perfect curve was obtained in that no males were produced in the highest concentration of bacteria while steadily increasing percentages of males were produced as the bacteria decreased in numbers in the medium. Curve I in Figure 2 gives the

results of such an experiment selected to show the possibilities that may be expected when conditions can be still more critically controlled. In these experiments no attempt has been made to standardize accurately the numbers of bacteria used in the different ex-

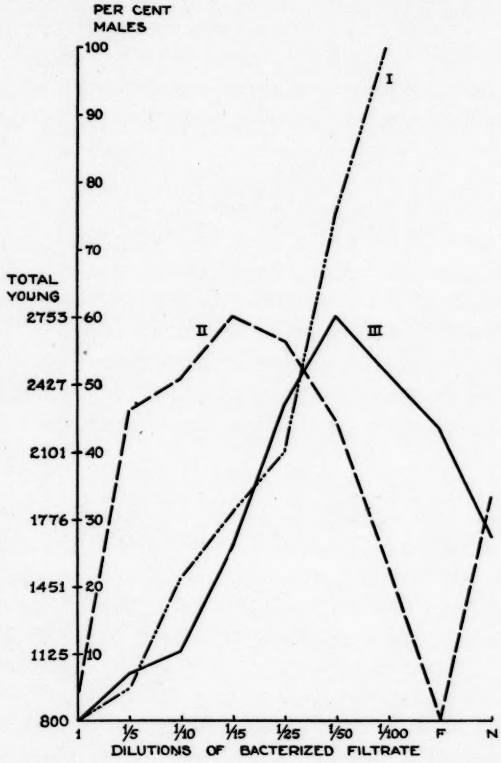

FIG. 2.—Relation between dilution of bacterized filtrate and number and sex of young produced. Curve I represents the male percentages in a single series; curve II, the total numbers of young produced in the different dilutions (22 experiments); curve III, male percentages (average for 22 experiments).

periments. It is realized that considerable variations occur in the suspensions since they were prepared by merely using the same number of slants for the different experiments, or, at best, attempt-

TABLE II

Exper. No.	Series of Bottles in Exper.	Dilutions of Bacterized Filtrate														Unbacterized Filtrate		Control—Normal Medium	
		1		1:5		1:10		1:15		1:25		1:50		1:100					
		Total No. Young	Per Cent ♂'s	Total No. Young	Per Cent ♂'s	Total No. Young	Per Cent ♂'s	Total No. Young	Per Cent ♂'s	Total No. Young	Per Cent ♂'s	Total No. Young	Per Cent ♂'s	Total No. Young	Per Cent ♂'s	Total No. Young	Per Cent ♂'s	Total No. Young	Per Cent ♂'s
4	4	122	0	182	0	177	3.4	168	31.6	150	81.3	111	95.5	36	100.0	.....	.....	.....	.....
8	4	167	0	205	0	213	5.6	206	34.0	153	60.8	139	94.2	63	85.7	.....	.....	.....	.....
10	2	68	0	72	0	60	16.7	64	25.0	55	60.0	47	100.0	31	100.0	.....	.....	.....	.....
14	4	55	0	83	0	113	0.9	196	10.2	185	6.5	189	19.1	185	11.9	.....	.....	102	24.5
18	4	14	0	79	0	72	5.6	77	0	46	6.5	62	56.5	63	19.1	.....	.....	12	8.3
22	4	38	0	57	17.5	25	0	50	32.0	70	44.3	51	86.3	37	100.0	.....	.....	120	33.3
26	4	77	0	86	0	108	19.4	140	7.9	165	52.1	101	36.6	104	51.0	.....	.....	199	11.6
30	4	95	0	252	19.1	238	32.4	220	80.9	206	90.8	113	100.0	47	63.8	.....	.....	188	19.7
32	2	87	0	90	0	91	0	104	0	91	35.2	80	53.8	61	57.4	.....	.....	96	0
35	3	0	0	0	0	0	0	24	0	114	45.6	119	75.6	107	59.8	.....	.....	94	30.9
38	3	6	0	66	0	79	0	82	11.0	21	33.3	20	100.0	45	22.2	.....	.....	0	0
41	3	106	0	100	63.0	73	43.8	95	87.4	80	98.8	75	66.7	63	50.8	79	76.0	85	29.4
44	3	69	0	169	0	172	33.7	170	32.4	160	23.1	143	51.1	34	0	0	0	146	17.1
46	2	16	0	65	46.2	62	37.1	25	52.0	50	100.0	25	76.0	24	83.3	6	100.0	69	36.2
48	2	0	0	104	0	96	0	97	0	105	18.1	97	70.1	30	0	3	0	80	33.8
50	2	0	0	27	0	52	0	45	35.6	14	100.0	28	100.0	18	100.0	9	77.8	69	49.3
52	2	3	0	68	0	63	6.4	42	28.6	53	79.3	40	85.0	42	57.1	0	0	50	24.0
54	2	0	0	31	0	69	0	90	11.1	61	0	103	18.5	23	0	33	33.3	113	29.2
56	2	0	0	53	30.2	86	8.1	86	40.7	101	16.9	84	53.6	64	50.0	5	40.0	64	32.8
64	8	12	0	219	0	270	0	372	27.4	393	53.4	294	66.0	230	84.8	69	82.6	177	42.9
66	2	0	0	96	0	94	0	110	18.2	108	39.8	108	49.1	58	89.7	24	37.5	71	63.4
70	4	0	0	219	0	240	0	290	0	259	30.5	227	31.3	184	34.8	145	8.3	150	30.0
Total number of young	.....	935	.....	2,323	.....	2,453	.....	2,753	.....	2,640	.....	2,256	.....	1,549	.....	373	.....	1,885	.....
																		Grand total young 17,167	
Average number of young per mother	.....	4.5	.....	11.1	.....	11.7	.....	13.1	.....	12.6	.....	10.7	.....	7.4	.....	3.9	.....	10.5	.....
Average per cent ♂'s	.....	.....	0	.....	7.2	.....	10.4	.....	26.1	.....	47.3	.....	60.1	.....	53.0	.....	44.0	.....	27.8

ing to standardize the suspension according to turbidity. Moreover, the filtrate alone may contain as high as 1,000,000 bacteria, or as low as 100,000 per milliliter.

Curve II, Figure 2, is a graphic representation of the total numbers of young produced by the mothers in the various dilutions. It will be noticed that an excess of bacteria (dilution 1) is almost as inimical to the production of young as is an insufficient number. In dilution 1 a total of 935 young were released, while in dilution 1:15, 2,753 were produced and in the unbacterized filtrate but 817 (computed by interpolation) were released. There seems to be a toxic substance in the concentrated suspension as the mortality of mothers in these bottles was 55 per cent higher than in the unbacterized filtrate. No attempt has been made to analyze the nature of the factor responsible for the high mortality in the heavily bacterized bottles. The small number of young produced in the filtrate, despite a low death-rate of mothers, can be ascribed to the facts that the clutches produced were usually very small, that some mothers in this environment never laid eggs into the brood chamber, some laid eggs which failed to develop, while some mothers produced only sexual eggs.

RETARDATION

Banta and Brown (1929*d*, 1929*e*) have observed that the production of males by Cladocera is accompanied by a retardation in the time of release of these young. Accurate times of release were not taken in most of these experiments, but the order in which the releases occurred in the various bottles of a series was noted, and a general conception of the relative amount of retardation can be gained by considering the sequence of releases in the dilutions. Mothers in dilution 1:5 always released before those in dilution 1. Following these, releases occurred in dilutions 1:10 and 1:15, respectively, with the normal control releasing usually after 1:10 and before 1:15. All these releases usually occurred within 2 hours from the time of the first release in any bottle. Releases next came in the 1:25 and 1:50 dilution, involving a delay of from 2–6 hours. Dilution 1:100 and the unbacterized filtrate next released in order with a retardation that sometimes reached as high as 14 hours. Curve III in Figure 2 shows, however, that following a maximum male pro-

duction in dilution 50 there is on the average actually a decrease in the average percentage of males produced in the next dilution (100) and in the unbacterized filtrate. This fact is extremely significant since bacterial counts show that there is no increase in the bacterial numbers in these bottles over the preceding dilutions—in fact, the count in the last dilution is proportional to the dilution (100) while that in the filtrate is considerably lower. Banta and Brown (unpublished manuscript) have suggested that retardation in the time of release of cladoceran mothers may up to a certain point be indicative of male production, but that excessive retardation is ordinarily followed by the production of females. This conception is in complete agreement with the results obtained from these experiments. It will also be noted that, although releases occurred in dilution 5 before dilution 1, males are found in the former and not in the latter. The retardation in dilution 1, as far as could be ascertained, occurs after the eggs are laid in the brood chamber and not during the critical period for sex determination, namely, about 4 hours before egg-laying (Banta and Brown, 1929*c*).

Relation of bacterial counts to the sex of young in the various dilutions.—Experiments were undertaken to establish the exact number of living bacteria present in the varying dilutions and to correlate the count with the sex of the young produced by mothers crowded in these dilutions. The bacterized filtrate dilutions were prepared in the manner previously described except that 11 ml. were placed in each small bottle and mixed more carefully than usual. One milliliter was then removed from each dilution and a bacterial count made on it after the method used in water analysis. All counts were made on agar plates after 48 hours' incubation at room temperature. As soon as the milliliter sample was removed from a bottle three mothers were introduced in each dilution as in a regular experiment. At the approximate time of egg-laying a second 1-ml. sample of the medium was withdrawn from each dilution and a count made as before. When the mothers released, the sex and total number of young released were determined. The results of these experiments (averages of three different series) will be found in Figure 3.

This figure shows that as the numbers of bacteria in the varying dilutions decreased the percentages of males produced in the varying

dilutions increased. One billion to 500,000,000 bacteria per milliliter in the bacterized filtrate resulted in the release of female young only. Approximately 70,000,000 bacteria per milliliter resulted in about 10 per cent males; while 13,000,000 produced 27 per cent males; and but 1,000,000 bacteria per milliliter gave 65 per cent males. The

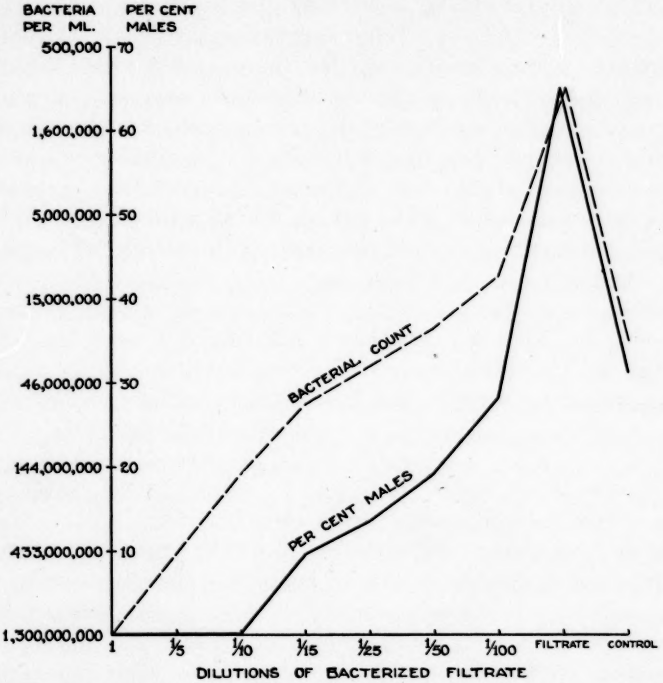

FIG. 3.—Relation between actual numbers of bacteria in medium and percentages of male young produced.

per cent males (18.75) produced in the dilution containing 27,000,000 bacteria does not correlate as well as was anticipated with the percentage of males (31.13) resulting from the normal control containing 25,000,000 bacteria per milliliter. One would expect to obtain a percentage of males in the control more closely approximating the percentage of males produced in a dilution of the bacterized filtrate containing about the same number of bacteria. The discrepancy be-

comes more pronounced when the second count (at the time of egg-laying) is compared with the first count made at the time the mothers were placed in the medium. The numbers of bacteria in dilutions from 1 to 100 and the filtrate decrease about 55 per cent while the count in the normal control drops but 36 per cent. This means that there is a greater number of bacteria present (16,000,000 per milliliter) in the control at the time of egg-laying than in the 50 dilution (10,000,000), consequently a smaller percentage of males would be anticipated in the control. It is quite possible, however, that perfect correlation cannot be obtained under the present experimental conditions. The majority of bacteria in the 50 dilution are of one species, a species which these experiments (and others to be reported in a subsequent paper) show can be utilized as food by Cladocera, while in the normal medium this species of bacteria is in the minority. And it is not improbable that in the 16,000,000 bacteria per milliliter in the normal medium there are some species which the Cladocera cannot use as food. Furthermore, a large number of spores will always be found in the normal medium, and presumably these would not serve as food for the Cladocera.

DISCUSSION

Grosvenor and Smith (1913) and Banta and Brown (1929*a*) observed that crowding cladoceran mothers in limited amounts of culture medium resulted in the production of male young. The effective agent causing this production of males was considered to be the accumulation of excretory products of the animals. Although bacteria were regarded as a suitable food for Cladocera, little attention was given to the numbers of bacteria present in the medium. Diluting the medium in which the Cladocera were to be cultivated with pond water was considered equivalent to diluting the amount of available food. Bacterial counts made on various dilutions of the concentrated medium in pond water as here reported show, however, that dilution up to a certain point, 10–15 times, is quite ineffective as a means for decreasing the number of bacteria present. It has been demonstrated that in varying dilutions (within limits) of the normal medium with pond water crowded mothers not only produced the same percentages of males but also approximately the same

number of young per mother in the different dilutions. It would seem reasonable to suppose, then, that there was an insufficient amount of bacterial protein available for crowded mothers even in the concentrated medium. In our experiments mothers crowded in normal medium (three mothers to 10 ml.) to which had been added suspensions of pure cultures of bacteria in ample quantity produced only female young. Moreover, by properly grading the number of bacteria present in a medium, crowded mothers are caused to produce males, the percentage of males depending on the number of bacteria present in the medium. Under these conditions, subject to the usual experimental variations, from 0 to 100 per cent males have been produced by crowded mothers.

Retardation seems to be very closely associated with male production by crowded *Moina macrocopa* mothers. Throughout the work reported in this paper retardation up to a certain point has been concurrent with the production of males. These experiments also substantiate the view that excessive retardation results in the production of female young.

The increase in the production of males in varying dilutions of bacterized filtrate has been shown to be concomitant with a decrease in the bacterial count of the dilutions. This would indicate that the amount of available food in the form of bacteria exercises a marked effect on sex control. Before any attempt is made, however, to explain the precise rôle of bacteria as food in the control of sex careful investigations should be undertaken to determine the relation of the number of bacteria in a medium to the metabolism of the mothers in that medium.

Early in the work it was considered that the bacteria in the filtrate formed a colloidal suspension which, bearing a negative charge, might adsorb excretory substances produced by the Cladocera and in this manner influence sex control. To test this hypothesis siliceous earth, blood, charcoal, kaolin, and other negatively charged substances were suspended in varying concentrations in normal medium containing crowded mothers. There was no decrease in the percentage of males produced in these experiments below that produced by sister-mothers crowded to the same degree in normal medium alone.

CONCLUSIONS

1. Sex of the young produced by experimentally crowded *Moina macrocopa* mothers can be controlled by the amount of available food (bacteria) present in the medium.

2. Retardation up to a certain point is concurrent with male production but excessive retardation is followed by the production of females.

3. The bacteria present in the medium do not appear to influence the control of sex by the adsorption of excretory substances.

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STUDIES ON BACTERIOLOGICALLY STERILE MOINA MACROCOPA AND THEIR FOOD REQUIREMENTS[1]

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MANY experiments involving the physiological activities of Cladocera have been conducted without the previous determination of the food requirements of these animals. It is generally conceded that unicellular algae, bacteria, and possibly protein débris from disintegrating plants and animals may serve as effectual food, but the relative merits of these different substances, or, more pertinently, the possible self-sufficiency of any one of them, has never been investigated so far as can be ascertained from the literature. It is the purpose of this paper to cover the last of these points in so far as it concerns bacteria, to interpret tentatively the rôle of protein débris as effectual food, and to propose bacteriological methods applicable to such investigations.

In a previous paper Stuart and Banta (in press) presented evidence in support of the contention that bacteria may serve as a satisfactory food for Cladocera. In these experiments paper filtrates of aged manure infusions, inadequate in themselves to maintain normal growth of Cladocera, when reinforced with suspensions of bacteria, became superior to the manure infusion commonly used for the routine cultivation of Cladocera. It was shown that mothers reared in a properly bacterized medium released sooner and produced about 20 per cent more young than their sister-mothers in normal medium. This evidence, while fairly conclusive regarding the effectiveness of bacterial proteins as food, does not take into consideration the relative food value of different species of bacteria present in or added to the medium. This is important since it is quite improbable that all species of bacteria will serve equally well as food for these animals.

[1] Grateful acknowledgment is made to the Department of Genetics of the Carnegie Institution of Washington for aid in the support of this work.

Moreover, having added living bacteria to the medium, no information could be acquired from these experiments pertaining to the food value of dead bacteria or bacterial débris.

In an effort to establish for *Moina macrocopa* the relative food value of different species of bacteria, mothers were reared in a medium initially containing but a single species of bacteria. The Cladocera used in these experiments were washed in several changes of sterile pond water in order to lessen by dilution the numbers of bacteria unavoidably carried over from the normal medium in which the animals were cultivated. The washed mothers were then placed in bottles (one, two, or three per bottle) containing 50 ml.[1] of sterilized normal medium which after sterilization had been inoculated with a suspension of *Bacillus coli mutabole* (1907). The growth of the experimental mothers in this environment was in every respect comparable to that of the control mothers in normal medium. It was hoped that the species of bacteria inoculated into the medium (*B. coli mutabole*) by reason of its advantage in numbers would outgrow any other species of bacteria that might be introduced with or by the Cladocera. A periodic bacteriological analysis of the bacterized medium, however, showed that in many bottles after 12–16 hours species of bacteria other than that inoculated were present in considerable numbers. In only a small percentage of the bottles after 48 hours was the inoculated species able to maintain its supremacy of numbers over the contaminating species. These experiments indicate that the value of a single species of bacteria as food cannot be determined with any degree of accuracy by this method. The fact that a mother reared in a bacterized medium thrives fully as well as a control mother in normal medium is presumptive evidence of the food value of a given species of bacteria for Cladocera, since in the first 24 hours in a bacterized bottle no species other than the one inoculated is present in sufficient numbers to support the growth of Cladocera. This does not exclude the possibility that some or all of the contaminating species may play some essential rôle during a later period of development of the Cladocera, such as, for example, the egg-laying period. It was, therefore, considered advisable to abandon this approach to the problem.

[1] Ml. = milliliters.

A bacteriological examination of the digestive tract of Cladocera (dissected out by Mr. C. D. Williams of this department) showed that the anterior end of the tract contained large numbers of living bacteria. Since the intestinal canals of these animals contained viable bacteria, any attempt to cultivate Cladocera in a medium containing but a single species of bacteria necessitates the development of a technic for securing bacteriologically sterile Cladocera. Stuart and Gorham (1925) found that sterilization of mosquito larvae by the use of chemical disinfectants was not feasible, but that sterilization could be effected by successive washings of the larvae in sterile distilled water, the series of washings extending over a period of approximately 24 hours.

APPARATUS AND MATERIALS

After a considerable number of experiments were made involving the type of water to be used for washing (distilled, tap, or pond water), the number of times the Cladocera should be washed, and the length of time the animals should remain in the successive wash waters, the following technic was found to be successful for rendering *Moina macrocopa* bacteriologically sterile. The animals are removed from the normal medium with a small pipette (care being exercised to avoid carrying over any more of the medium than necessary) and placed in a flask containing 500 ml. of autoclaved pond water. The flask is rotated gently a few times, and after 15 minutes in this water the animals are transferred to a second flask containing 500 ml. of sterile pond water. This procedure, repeated four times at 15-minute intervals, serves to dilute out the gross contamination carried over from the medium with the Cladocera. After the preliminary washings from one to three animals are placed in a 250-ml. cotton-stoppered dilution bottle containing 150 ml. of sterile pond water. One animal to a bottle insures a greater percentage of sterile Cladocera after the necessary washings, but three animals to a bottle results in a saving of time and materials which is more important especially when large numbers of sterilized animals are needed for experimental purposes. The animals remain in the first dilution bottle for 1 hour and are then transferred to a similar bottle where they are again allowed to remain for 1 hour before being transferred

to a third bottle. The number of times the Cladocera should be washed in this manner depends to some extent on the number of sterile animals required for any one experiment. It was found that ten such washings with three animals to a bottle resulted in from 40 to 50 per cent of the animals being sterile, while fifteen washings sterilized from 80 to 90 per cent of the animals. Sterile pipettes are, of course, essential, but it was found advisable for this work to discard the nipple usually attached to the pipette. By holding the index finger tightly over the top of the pipette the delivery end is brought close to the Cladocera, then by suddenly releasing the finger the animals are drawn into the pipette. By the proper manipulation of the pipette one is frequently able to secure all three animals from a bottle at one time and so to diminish the risk of contamination. Cladocera, approximately 8–12 hours old, are best for sterilizing purposes. Younger animals too frequently fail to survive the number of washings required to sterilize them, and older animals seem to require a much longer period of washing than those in their first instar. Possibly a molt during the process of sterilization is essential to eliminate the bacteria adhering to the surface of the cladocerans.

In the course of the first few experiments in sterilizing Cladocera it was noted that sodium silicate crystals were frequently formed in autoclaved pond water, owing doubtless to the inferior quality of the glass of the dilution bottles. Hydrogen-ion titration of pond water in a series of bottles after successive sterilizations gave these readings: before sterilization, pH 6.8–7.0; after sterilization, pH 7.3–7.5; after two successive sterilizations, pH 8.0–8.5; and after three successive sterilizations, pH 8.9–9.4. Further sterilization had but little effect on the hydrogen-ion concentration.

It seemed improbable that Cladocera could survive for any considerable period of time in a medium as alkaline as pH 9.5; and before proceeding further with the work the following experiment was tried. Three lots of thirty Cladocera each were washed in the manner previously described. (The use of three series of bottles, thirty to a series, greatly facilitates the work, for when the animals are transferred from one bottle of a series to another of the second series the used bottle may be resterilized or refilled and sterilized. Three series of the bottles are necessary as it is impossible to refill, sterilize,

and cool one series in time to be used for the next transfer of the Cladocera.) The *Moina* of the first lot of thirty were washed (three to a bottle) fifteen times in bottles which after being used for one washing were emptied, rinsed, refilled with pond water and sterilized. By this procedure the animals were carried for 15 hours in pond water with a hydrogen-ion concentration of approximately 7.4. The thirty Cladocera of the second lot were washed (three to a bottle) fifteen times in bottles which after being used for one washing were resterilized without refilling; but after being used a second time were emptied, rinsed, refilled, and sterilized. Then since three series of bottles were used, each series was refilled three times and the Cladocera in this lot were carried for 9 hours in pond water with a hydrogen-ion concentration of approximately 7.4 and for 6 hours at approximately 8.3. The Cladocera of the third lot of thirty were washed (three to a bottle) fifteen times in bottles which were not refilled at any time during the washings. The animals, then, were in pond water, pH 7.4, for 3 hours, pH 8.3 for 3 hours, and pH 8.9–9.4 for 9 hours. At the conclusion of the experiment the three lots of Cladocera so sterilized showed no significant differences. All had about the same percentage of sterile animals, the same degree of vitality as far as could be observed, and there were no deaths in any of the three lots. This does not signify that Cladocera can be maintained in any medium with an alkalinity of pH 9.4 since, as later experiments proved, had these same animals been placed in a highly buffered, sterile, bacteriological broth of pH 8.0 they would have died in about an hour; whereas placed in broth pH 7.0 many would have survived to produce young.

In every experiment after the successive washings different numbers of the Cladocera were tested for sterility. Generally this was accomplished by transferring the animals from the last wash water to tubes (one animal to a tube) containing from 6 to 25 ml. of double proteose-peptone broth. The broth was prepared by boiling 20 grams of proteose-peptone and 6 grams of beef extract into solution in 1,000 ml. of distilled water, titrating to pH 7.0–7.2, filtering and sterilizing. In all experiments the broth tubes with the Cladocera were incubated at room temperature for at least two weeks in order that any delayed growth of bacteria might be observed. A clado-

ceran in any tube showing evidence of bacterial growth was considered contaminated. It is doubtful whether the results of this procedure showed the number of Cladocera that were actually sterilized in any experiment since the time required to capture and transfer an animal from the last wash water to the broth afforded ample opportunity for extraneous contamination of wash water or broth. There being no way, however, to distinguish between air-borne contamination and that contributed by the animals, it is imperative that any growth in the broth be considered as coming from the Cladocera. Earlier in the work the presence of anaërobic bacteria in the washed Cladocera was investigated. Ten Cladocera washed twelve times were introduced into ten tubes of mashed veal and broth stratified with a layer of petroleum oil to exclude oxygen. The same procedure was repeated with the débris of ten similarly washed Cladocera that were crushed with a sterile glass rod. Growth occurred in four of the twenty tubes. In two of the tubes showing contamination growth appeared in 24 hours and the organisms present were found to be facultative spore-formers commonly present in air. Growth in the other contaminated tubes did not appear until the fourth day. The organisms proved to be pure cultures of granular, non-spore-forming, straight rods which grew luxuriantly in an anaërobic environment, but very poorly under aërobic conditions. This species of bacteria was frequently found as the contaminating species in the sterility tests of washed Cladocera, though its presence in many tubes could not be detected until the fifth or sixth day of incubation.

As a further test for the presence of anaërobes the crushed bodies of fifteen Cladocera, each with 1 ml. of the broth in which the Cladocera had remained without visible bacterial growth for 7 days, were pipetted into fifteen sterile petri dishes which were then poured with agar and incubated at room temperature for 2 weeks in an atmosphere of hydrogen in a Novy jar. Growth occurred in 48 hours on two of these plates. The species present on both plates, *Bacillus subtilis*, was held to be air-borne contamination doubtlessly introduced at the time of the transfer of the Cladocera and broth to the plates since growth of this species would have been apparent in the broth before plating had these organisms been present in the broth. With twelve more washed Cladocera the same procedure was repeat-

ed except that the plates were incubated in a Smillie jar. All twelve plates were sterile. From these experiments it may be concluded that following sufficient washing strictly anaërobic bacteria are rarely if ever present in the Cladocera and, therefore, conclusive sterility tests of washed animals may be made in broth medium under aërobic conditions provided 14 days' incubation is allowed. This is important as it simplifies the work considerably. It is admitted that these tests do not exclude the possible presence of obligatory parasitic bacteria which would fail to grow apart from the living Cladocera. Even were such organisms present it is difficult to see how they could in any way influence the conclusions drawn in this paper. In eleven different experiments more than one thousand *Moina macrocopa* mothers were sterilized in the manner described. As controls the sterility of three hundred and thirty-six of these mothers was tested in broth and of these 77.7 per cent proved to be sterile. The remainder were used in various experiments, involving in every case an indirect or delayed test for sterility and approximately the same percentage of these was found to be sterile.

SOLUBLE PROTEINS AND BACTERIA AS FOOD FOR CLADOCERA

Before any attempt was made to test the relative nutritive value of different substances it seemed advisable to study the behavior of sterile Cladocera in a medium devoid of any possible nutritive substances. Washed Cladocera were placed in tubes (one Cladocera to a tube) of sterile distilled water and their behavior and general appearance noted.

Another series of washed Cladocera was placed in the following autoclaved media: tap water, pond water, and normal medium; and in addition in an unheated sterile Berkefeld filtrate of normal medium. The behavior, general appearance, and death or releases of the animals in these media were noted. The Berkefeld filtrate is particularly important in this series since it will show whether autoclaving the normal medium destroys by coagulation any soluble protein substances that might otherwise serve as food for the Cladocera. All the tubes with the exception of the broth tubes were tested for sterility at the end of 2 weeks by transferring the Cladocera

and 1 ml. of the medium to 25 ml. of broth. In a second experiment *B. coli mutabole* were added to the same types of medium which were subsequently sterilized in the autoclave. *B. coli mutabole* were also added to a Berkefeld filtrate of normal medium and killed by heating at 56° C. for 1 hour. Cladocera were placed in tubes of each of these media and observed and tested for sterility as in the first experiment. The tabulation of these experiments shown in Table I includes only those Cladocera that by the broth test proved to be sterile.

As will be seen in Table I, all the Cladocera in the distilled water and tap water died without releasing young and as far as could be

TABLE I

SOLUBLE PROTEINS AND DEAD BACTERIA AS FOOD

Unbacter. Medium Ster'd	No. Anim.	% Rel'g Young	Aver. No. Young Rel'd	Bacter. Medium Ster'd	No. Anim.	% Rel'g Young	Aver. No. Young Rel'd
Distilled water....	11	0	0	Distilled water....	12	50	4
Tap water.....	5	0	0	Tap water.....	12	41.6	5
Pond water.....	5	27.2	4	Pond water.....	11	36.3	4
Berk. filter, normal medium.....	16	33.3	5	Berk. filter, normal medium.....	24	54.1	3
Normal medium...	13	40	5	Berk. filter, normal medium heat 56° C.....	16	41.2	5
Broth.....	336	39.5	6	Normal medium...	32	50	6
				Broth.....	11	45.4	5

ascertained without laying eggs. They did not appreciably increase in size, though up to the time of death (about the fourth day) their vitality and vigor were not visibly impaired. In the other types of media all the Cladocera grew noticeably and their intestinal canals, which were colorless after the animals had been washed, grew perceptibly darker. About 35 per cent of these Cladocera released an average of five young per mother. In the distilled water, bacterized with killed bacteria, 50 per cent of the Cladocera released an average of four young; in the tap water 41.6 per cent released an average of five young; whereas in each of the other types of media so bacterized a slightly higher percentage of releases occurred than in the corresponding unbacterized medium.

It is significant that no medium bacterized with killed bacteria supported continuous growth, i.e., the production of successive broods or successive generations, of the cladocerans whether the bacteria were killed by autoclaving or by heating to 56° C. for 1 hour. The latter method of killing bacteria does not employ sufficient heat to alter to any extent the protein nature of the bacteria, since after such sterilization the proteins retain their full antigenic capacity and serologic specificity. Yet bacteria killed in this manner although they remain suspended in the medium are not effective as food for *Moina*. It will be shown later in this paper that *Moina* can be cultivated indefinitely on this same species of bacteria when it is added to a medium in a viable condition. It seems, therefore, that bacteria in order to serve as effective food for Cladocera must be supplied in a viable condition.

The following experiments were undertaken to determine the relative food value of different species of living bacteria. Eleven species of bacteria taken from 48-hour agar-slant cultures incubated at room temperature were suspended in 5 ml. of sterile pond water. With aseptic technic these suspensions were added to tubes containing 25 ml. of sterilized normal medium, in quantities so that all tubes contained an amount of suspension equivalent to 0.1 ml. of an *Aerobacter aerogenes* suspension. A single washed young female *Moina macrocopa* was then placed in each tube of the bacterized medium and observed from day to day. As a control washed females were placed in tubes (one to a tube) of normal medium. The number of young released by the mothers in the different tubes was noted as well as any deaths that occurred. When a mother released or died in any tube 1 ml. of the medium from that tube was diluted as in water analysis and plated in order that the presence of any organisms other than the species inoculated might be detected. Both standard agar and Endo's agar were used for this work. Endo's agar was found to be very advantageous when working with lactose-fermenting bacteria such as *B. coli mutabole* and *A. aerogenes*. In some of the experiments, when a mother released, one of the young was sterilely transferred to a previously prepared tube of the same medium. In this way the reaction of successive generations of Cladocera to a single species of bacteria could be followed. For example:

eleven washed mothers were placed in eleven tubes of sterilized normal medium bacterized with *A. aerogenes*. Ten of these eleven mothers released young. A single offspring from eight of the ten mothers that released was sterilely transferred to a freshly prepared tube of the same medium and the tube containing the mother tested for the presence of any organism other than *A. aerogenes*. Seven of the eight young transferred released (one contaminated) and a single offspring from five of the seven that released was transferred as before and the proper platings made to determine the presence of any contaminating species of bacteria. This was repeated for six filial generations from the original mothers. The results of this and other similar experiments are recorded in Table II.

As shown by Table II, *Moina macrocopa* mothers do not thrive equally well on all species of bacteria. Of the eleven species and one experimental mixture used *B. coli mutabole* and *A. aerogenes* seem to provide the most satisfactory nutritive material for *Moina*. More animals survived, and they produced larger broods of young when cultured on these two species than on any other species of bacteria used in the experiments. It is interesting to note that washed mothers on their first release do not produce a normal-sized clutch regardless of the medium in which they are placed. Presumably certain of the normal biological activities are disturbed by the long series of washings required to produce sterile animals. Second clutches are usually normal in size. Washed mothers reared in pure cultures of *B. coli mutabole* appear to be in a better environment than washed mothers in normal medium. Of sixty-eight washed mothers reared in normal medium 75 per cent, or fifty-one animals, released in their first clutch an average of six and two-tenths young while of forty-six washed mothers reared in *B. coli mutabole* bacterized medium 95.6 per cent, or forty-four animals (one contaminated), released an average of seven young. Certain of the species of bacteria used in these experiments not only failed to support the growth of Cladocera but seem to be toxic to them. Predominating numbers of *Chromobacterium violaceum* in any medium are fatal to *Moina*. The toxicity of this species is clearly associated with its pigment since non-pigmented strains of the same species can be utilized by Cladocera as food. This also appears to be true for highly pigmented strains of

TABLE II

SPECIES OF BACTERIA AS FOOD

Species	Gen. Reared	No. Separate Exper.	Total No. Anim. Tested	Died	No. Anim. Rel'g Young	Aver. Young per Clutch	% Anim. Prod'g Young	% Living Animals Uncount'd
Normal medium.	1	5	68	17	51	6.2	75	.....
	2	3	14	3	11	7.6	78.5	.....
	3	2	3	1	2	10.5	66.6	.....
Bacillus coli mutabole.....	1	4	46	1	44	7	95.6	97.8
	2	4	25	11	13	8	52	92.9
	3	2	3	2	1	10	33.3	100
	4	1	1	0	1	14	100	100
	5	1	1	0	0	0	0	0
Aerobacter aerogenes.....	1	2	11	1	10	3.3	90.9	100
	2	2	8	0	7	10.7	87.5	87.5
	3	2	5	2	1	12	20	33.3
	4	1	1	0	1	8	100	100
	5	1	1	0	1	17	100	100
	6	1	1	0	1	15	100	100
Staphylococcus albus.....	1	1	6	1	4	1	66.6	80
	2	1	3	0	3	8	100	100
	3	1	2	0	2	10	100	100
Alkaligenes fecalis	1	1	6	1	4	2	66.6	80
	2	1	4	1	2	9	50	66.6
	3	1	2	2	.....	.....	.....	No test
B. subtilis.....	1	1	6	4	0	0	0	0
Sarcina luteus...	1	1	6	3	3	1	50	100
	2	1	3	0	3	8	100	100
	3	1	3	0	3	7	100	100
Spirillum rubrum	1	1	5	1	4	6	80	No test purity
Chromobact. violaceum.....	1	1	6	6	.....	.....	.....	6 pure
Unknown 1*....	1	1	6	6	.....	.....	.....	2 pure
Unknown 2*....	1	1	6	6	.....	.....	.....	3 pure
Unknown 3*....	1	1	6	3	3	2	50	100
	2	1	2	1	1	10	50	100
	3	1	1	1	.....	.....	.....	1 pure
Mixture.....	1	2	16	13	3	5	18.7	No test

* Isolated from normal medium.

various species of the genus *Serratia*. *B. subtilis*, the only spore-former used in these experiments, does not seem to be a satisfactory food.

DISCUSSION

A technic for rendering Cladocera bacteriologically sterile has been developed in these experiments and the relative value of soluble proteins, protein débris, and dead and living bacteria as food for *Moina macrocopa* has been investigated. Sterile Cladocera placed in sterile, unbacterized, distilled, or tap water die without evidencing any signs of growth and without releasing young. On the other hand, many sterile mothers placed in sterile bacterized (killed bacteria) or unbacterized pond water, broth, normal medium, and Berkefeld filtrate of normal medium showed growth and released young: albeit the clutches were small and no mother survived to release a second clutch nor did any survive to release a first clutch. In sterile distilled and tap water bacterized with killed bacteria, however, *Moina macrocopa* mothers grew appreciably in size, laid eggs, and released young.

The results of these experiments suggest that the mothers in those media where releases occurred had utilized soluble proteins, protein débris, or dead bodies of bacteria as food. Though we are inclined to believe that this interpretation is correct, the question is by no means satisfactorily answered in these experiments. If the idea that the Cladocera utilize the soluble protein, débris, or dead bacteria as food is rejected then only one other explanation is tenable, namely, that in the short time (10–12 hours) during which the Cladocera were in the normal medium before washing they stored a sufficient reserve supply of food to permit them to grow, lay eggs, and produce young. If this alternative is accepted one is forced to concede that the failure of the sterile Cladocera to grow or release in the unbacterized distilled and tap water is due to the hypotonicity of these solutions. It is not the purpose of this paper to attempt any explanation of these matters. It is realized that except for the bacteriological aspects of these experiments, i.e., the sterilizing of Cladocera, the testing of their sterility and rearing them on pure cultures of bacteria, any conclusions regarding the general biological activities of Cladocera must, for the lack of sufficient data, be inconclusive.

As was anticipated, not all species of bacteria can be utilized as food by the Cladocera. Of the eleven species and one mixture used in these experiments the two gram-negative intestinal bacteria *B. coli mutabole* and *A. aerogenes* were most satisfactory. This is not surprising since the manure infusion devised by Banta (1921), which affords an excellent medium for the routine cultivation of Cladocera, harbors an abundance of this general type of bacteria. Brilliantly pigmented strains of species from the genera *Chromobacterium* and *Serratia* (1930) prove to be toxic to the Cladocera. Species of these genera are commonly found in pond water, and their presence here in varying numbers may account for some of the variations often encountered in different batches of manure and soil infused in pond water. This does not imply that all chromogenic species of bacteria are toxic to Cladocera since Unknown 3 (Table II) isolated from the normal medium has yellow pigment (genus *Flavobacterium*, species unknown), yet it will support Cladocera for at least three generations.

These experiments were started at the suggestion of Dr. A. M. Banta, whose enthusiastic interest and support has in no small measure been responsible for carrying the work beyond the development of a technic for bacteriologically sterilizing Cladocera.

CONCLUSIONS

1. *Moina macrocopa* mothers can be rendered bacteriologically sterile by sufficiently prolonged and repeated washings in sterilized pond water.

2. Obligatory anaërobic species of bacteria do not seem to be present in washed Cladocera.

3. Sterile soluble proteins, protein débris, and dead bacterial bodies appear to sustain the growth of Cladocera, but only for a time and to a limited extent.

4. For the normal growth of *Moina* living bacterial cells are required as food.

5. All species of bacteria do not serve equally well as food for Cladocera.

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CHANGES IN THE OVARY AND OESTRUS CYCLE FOLLOWING THE REMOVAL OF ONE OVARY IN ALBINO RATS

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LONG ago Bond (1906) called attention to the hypertrophy of the remaining ovary after one ovary had been removed. Bond's observation has not only been confirmed by several investigators but in addition the cause of the hypertrophy has been more carefully considered. The effect of this hypertrophy on the oestrus cycle seems to have been neglected in the data found in the literature. Therefore, the experiments herein reported were undertaken to study the oestrus cycle after the removal of one ovary.

LITERATURE

The experimental literature began with Bond's (1906) description of ovarian hypertrophy in the rabbit. His experiments were carefully performed but led to the erroneous conclusion that copulation was necessary for hypertrophy of the surviving ovary. That copulation was not necessary for ovarian hypertrophy in the rabbit was shown by Carmichael and Marshall (1908) and in various other animals by all subsequent investigators. Likewise in the rat, if one ovary is removed the remaining ovary becomes enlarged (Hatai, 1913, and Stotsenburg, 1917) and contains many ripe follicles. According to Arai (1921), the large number of these well-developed follicles and corpora lutea are responsible for the increase in weight of the surviving ovary. This abundant follicular development was thought by Lipschutz (1928) to be brought about by some substance in the blood which he spoke of as an x substance. The work of Zondek and Aschheim (1927) and Smith and Engle (1927) on the gonad-stimulating hormone of the anterior pituitary gives evidence that this hormone is responsible for the hypertrophy of the surviving ovary. A careful review of the literature and a study of pituitary grafts into unilateral ovariectomized rats have been made by Engle

(1928). He states that the anterior pituitary furnishes the stimulus for the hypertrophy of the surviving ovary. Therefore, from the present literature it seems that the x substance of Lipschutz is the gonad-stimulating hormone of the anterior pituitary.

EXPERIMENTAL METHODS

The animals used in these experiments were as far as could be judged normal nullipara albino rats fed on a diet of ground grains and green food. Since the experimental and control animals were fed and caged in the same manner the care of these rats need not be further discussed. Ages and weights of all animals studied were recorded. The ovary was removed by dorsal laparotomy under ether anesthesia and the wound closed with sterile silk sutures. Litter mates of these operated rats were used as normal control rats.

The oestrus cycle was determined by daily vaginal smears obtained by the use of a small pipette and a drop of 0.7 per cent sodium chloride. This method was compared to the scrapings obtained from the vagina, by means of a small loop of wire, and found to be satisfactory and time saving.

EFFECT ON THE OESTRUS CYCLE

As has been briefly reported (Emery, 1930), the oestrus cycle is slightly shorter in rats after one ovary is removed. It was often found confusing to determine the duration of the cycle, owing to the fact that some of the rats with surviving ovaries were in continuous oestrus for several consecutive days. This led to a more detailed analysis of the data in respect to the number of consecutive days these rats were in oestrus. The types of vaginal smears have been classified according to the number of cornified cells present. A similar method has been used by others (Kahnt and Doisy, 1928, and Kennedy, 1930) and gives the type of information most useful in a comparative study of the type shown in Table I. Dioestrus may be considered to be a period of mostly leucocytes in the smears. If a few epithelial or cornified cells are present the smear is called scales + and considered to belong to dioestrus. A considerable number of cornified cells is called scales + +, a greater number + + +, and a maximal number of cornified cells as + + + +. In a few cases an exceptionally heavy smear may be spoken of as scales + + + + +.

This method has been found to be very applicable in observing a large number of animals in a short time. It has been observed that animals with scales + + will sometimes, and those with scales + + + or more will usually, accept the male. Therefore, scales + + or more are considered the oestrus period and represent the cornified cell stage shown in Table I. The epithelial cells have not been classified separately but have been considered as adding to the rating given the cornified cells. This method, though not as precise

TABLE I*

Consecutive Number of Days	Less than 5	5	10	15	20	25	30	35	40
Oestrus or Cornified Cell Stage									
A	59	39	0	1	0	1	0	.....	.....
B	21	33	16	9	7	10	2	2	0
Dioestrus or Leucocyte Cell Stage									
A	30	35	18	10	5	2	0	.....	.....
B	25	38	25	3	5	1	1	2	0

* Frequency distribution of the maximal number of consecutive days in oestrus and dioestrus of A, 100 normal rats; B, 100 semicastrated rats. These data are based on daily vaginal smears taken over a period of two to eight months.

as the method used by Kahnt and Doisy (1928), is considered adequate for a comparative study of large numbers of rats.

It is interesting to note that normal rats are rarely in heat more than 5 consecutive days (Table I), while the rats with the surviving ovary were often in heat 5 days or longer. Sixteen per cent of the latter were in oestrus 10 consecutive days, 9 per cent 15 days, and 2 exceptional cases 35 days. Only the maximum duration of oestrus is shown. Thus each rat is recorded only once in Table I, although there may have occurred several shorter periods of oestrus in the same animal while under observation. As shown, also in Table I, the normal and rats with surviving ovaries are almost identical in respect to the number of consecutive days in dioestrus. In some rats, however, there are irregular cycles with longer dioestrus periods

immediately following the operation, but these cases return to a more regular type within a few weeks. Likewise, crushing the uterus produced only a temporary change in the oestrus cycle (see series F 2, Table II).

In the normal rat the average length of time occupied by the oestrus period was about one-third of the total cycle. On the basis of a 5-day cycle, or 120 hours, the normal rat would be in oestrus about 36 per cent of this time (Table II), or about 43 hours. This is

TABLE II*

Series	No. Rats	No. Days Considered	No. Days in Oestrus	Percentage of Time in Oestrus			Age at Start, in Months	Remarks
				High	Low	Mean		
A	20	60	33.8	83	23	56.4	8	One ovary removed and these data started about two months later
B	10	60	36.7	90	41	61.2	8	
C	20	60	25.1	69	23	41.8	6	
D	10	120	37.5	57	16	31.3	3½	
F1	25	90	39.6	83	26	44.0	8	
F3	15	150	68.8	76	16	45.9	3	
E	10	60	26.7	56	35	44.5	6	Both ovaries partly removed two months before
E	10	60	22.0	53	17	36.7	2	Normal rats
F1	25	100	36.6	53	24	36.6	2	
F2	25	100	36.2	46	25	36.2	2	
F4	15	60	22.4	46	13	37.4	3	
F2	25	90	32.0	56	20	35.7	8	Uterus crushed and data started two months later

* The percentage of time in oestrus is shown for semiovariectomized rats and for normal controls. Those in series F1 and F2, also F3 and F4, are litter mates. Data were obtained from series E, F1, and F2 as normal rats, then these were operated on as indicated and data continued.

a slightly longer time than that given by Long and Evans (1922) for the period in which normal rats will mate. This period of 43 hours is thought to represent stages 2 and 3 and to extend into stages 1 and 4 as described by Long and Evans.

After one ovary has been removed and time allowed for the surviving ovary to hypertrophy the cornified cells may extend over a longer period of time. The percentage of the time these animals are in oestrus is variable and ranges from about 20 to 90 per cent of the total cycle (Table II). It will be noted that the mean percentage of

time in oestrus, except series D, is considerably above the 36 per cent characteristic of normal rats. The rats in series D were operated on when about one month old; this was at first thought to account for these rats being only 30 per cent of the time in oestrus. To test this idea the rats in series F_3 were operated on when one month old and oestrus smears taken in the usual way. These data (Table II) show that series F_3 averaged about 46 per cent of the time in oestrus, as compared to about 36 per cent for the normals.

TABLE III*

	Number of Ovaries	Mean Weight	Per 100-Gram Body Weight	
			Mean Wt. of Ovaries	Per Cent Increase above the Controls
Surviving ovaries	25 left	40.0	22.7 ± 0.77	73
	75 right	35.0	19.6 ± 0.42	44
Average	100†	36.5	20.4 ± 0.37	52
Normal rats (controls)	25 left	23.5	13.1 ± 0.39	
	75 right	21.8	13.6 ± 0.31	
Average	100†	22.2	13.4 ± 0.25	

* The weights in milligrams of one hundred surviving ovaries are compared to the weights of ovaries of normal rats. The surviving ovaries were removed three to twelve months after semiovariectomy.

† Total.

Thus, it is evident that the removal of one ovary at an early age did not account for the low percentage shown by the rats in series D. Neither was there any reason to suspect that the health or any other factor now known was responsible. Series D is, therefore, placed in the table to show the variations that may occur in rats with surviving ovaries.

CHANGES IN THE OVARY

The increase in weight of the surviving ovary following semicastration is 52 per cent, as seen in Table III. The average weight of the normal and surviving ovaries per 100-gram body weight are also

shown in Table III. All these rats were six months of age or older when killed but the body weights differ considerably due to the condition of the animal. The body weights of adult rats appear to have little to do with the weight of the surviving ovary, which is probably more closely related to the hormone of the anterior pituitary and the large number of well-developed graafian follicles (Engle, 1928, and Arai, 1921). The variability in weight is further shown by the probable error which is also quite large even when one hundred ovaries are considered (Table III). Since the ovaries were carefully removed and weighed in closed containers, the error due to weighing for such a large number of cases is very small.

The difference between the weight of the right and left ovary is slight. The data from 25 pairs of ovaries show the average weight of the right ovary to be 98 per cent of the left in normal rats. This result compares unfavorably with Arai (1920), who found that the weight of the right ovary of rats was 90 per cent of the left.

Sections of surviving ovaries showed that many corpora lutea and large follicles are largely responsible for the increase in size. The number of follicles and corpora lutea of such ovaries has been so thoroughly reviewed by many workers (Bond, 1906; Arai, 1921; Slonaker, 1927) that these need not be further discussed. Cysts of varying sizes were found in one-half or more of the hypertrophied ovaries and only occasionally in ovaries of normal rats. Some of these cysts were easily seen at autopsy while others were found only by microscopic examination. All cysts were more numerous eight to twelve months after one ovary was removed. Bond (1906) long ago called attention to these cysts in surviving ovaries, but recent investigators have almost entirely neglected them. Lipschutz (1928) found cysts in remaining ovarian fragments of the cat, and Wang and Guttmacher (1927) reported cystic formation following trauma of the ovaries of rats. The writer has observed that cystic ovaries frequently are found in semicastrated rats showing daily a large number of cornified cells, but such cells may occur without cystic ovaries, also cystic ovaries may be found in animals showing normal cycles at the time they were killed. It would seem from these observations, and from the fact that cysts are frequently produced by pituitary grafts in adult rats (Smith and Engle, 1927), that the

large follicles and cysts of surviving ovaries are the result of the gonad-stimulating hormone of the anterior pituitary.

DISCUSSION

There seems to be agreement at the present time that the gonad-stimulating hormone of the anterior pituitary in some way stimulates growth in the surviving ovary, after one ovary has been removed. The work of Arai (1921) and Lipschutz (1928) points to the follicles and corpora lutea rather than the interstitial tissue for increased development resulting in hypertrophy of the ovary. This is in agreement with the numerous reports coming out on pituitary grafting and injecting in mammals (Smith and Engle, 1927; Engle, 1928; Aschheim, 1930). Just how the removal of one ovary affects the pituitary is not clear. Engle (1928) considers the remaining ovary as utilizing the gonad-stimulating hormone previously used by both ovaries. A similar explanation could be applied to the increased weight of the ovarian fragments observed in the cat by Lipschutz and Voss (1925). Another way in which the hypertrophy of the ovary might be accounted for is by increased activity on the part of the pituitary. It has been reported (Hatai, 1913) that the pituitary does not increase after semiovariectomy but does increase in weight after complete castration in either male or female. Also Engle (1929) and Evans and Simpson (1929) have shown that the pituitary of the castrated rat was more effective in stimulating growth of immature ovaries than was the pituitary of the normal rat. It would be interesting to know if the anterior pituitary sex hormone of the semiovariectomized rat is increased in potency. A number of experiments have been carried out in this laboratory on pituitary grafting and testing the blood of such rats. This work is not completed at this time, but it may be of interest to state that little or no difference has been found in the potency of pituitaries from normal and semiovariectomized rats.

The irregular oestrus cycles with a tendency for increase in length of the cornified cell stage is very characteristic of semiovariectomized rats and is seen more frequently four to eight months after the operation. In a group of rats (series F_3, Table II) in which the ovary was removed at one month of age these irregular cycles become quite

pronounced about eight months after the operation. It seems, therefore, that the ovary must hypertrophy before there can occur any great prolonged period of oestrus. Some of these rats one year after one ovary was removed still showed many cornified cells present daily in the vaginal smears. These irregular periods frequently cease and normal cycles occur for a time. As mentioned in a previous section, the ovaries are cystic in about 50 per cent of these rats and this may have quite an effect on the oestrus cycle. Some of the rats with irregular cycles have been found at autopsy to have cystic ovaries, while in other cases the ovary is enlarged and cystic but the cycles are fairly normal. The greatest number of cysts was produced by removing part of both ovaries (series E, Table II), but the vaginal smears of these rats were not particularly different from those of rats with surviving ovaries. The rare occurrence of prolonged periods of cornified cells in normal rats, as pointed out in a previous section (see also Evans and Long, 1921), makes these changes in semiovariectomized rats all the more striking.

The increase of 52 per cent (Table III) in the weight of the surviving ovary is not as great as was expected, for Hatai (1913), Stotsenburg (1917), and Arai (1921) had shown that the surviving ovaries of rats were double in weight. It also compares unfavorably to Bond (1906) and to Carmichael and Marshall (1908), who found the weights doubled in surviving ovaries of rabbits. The ovaries (Table III) were dissected free from fat and visible cysts before weighing; yet the increase in weight of the surviving ovaries is smaller than that reported by Stotsenburg (1917) who did not include the cystic ovaries in the hypertrophied group.

Reproduction in animals with only one ovary has been well reviewed by Hanson and Boone (1926) and Crew (1927). The reproduction is apparently normal except for a slightly smaller number of young per litter. In connection with the studies on the oestrus cycle it was found that many of the rats with enlarged ovaries did not become readily pregnant when mated. This is often seen in nullipara six months or longer after the operation. Perhaps a more detailed study along this line would bring out additional differences in rats with enlarged ovaries. It would be interesting to know if copulation occurring during pregnancy, as reported by Nelson (1929), has any connection with enlarged or cystic ovaries.

SUMMARY

1. Semiovariectomized albino rats have been studied in respect to the oestrus cycle and the hypertrophy of the surviving ovary.

2. About two months after the operation the surviving ovary is increased in weight and irregular oestrus cycles begin to appear.

3. The cornified cells of oestrus continued for many consecutive days in some of these rats.

4. Ovarian cysts were found in about 50 per cent of the surviving ovaries removed three to twelve months after the operation.

5. The relationship of the gonad-stimulating hormone of the anterior pituitary to these changes is discussed.

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STUDIES ON TRANSPLANTATION IN PLANARIA

(Seventy figures)

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INTRODUCTION

DURING the last three years the writer has been engaged in a study of transplantation in *Planaria*. The object of this research was, first, to determine the effect or influence of the graft on the host and of the host on the graft; and second, to determine how the different levels or regions of the body of *Planaria* differ (*a*) as host and (*b*) as graft. The investigation was suggested by Dr. C. M. Child, and the work has been carried on under his direction. The writer wishes to acknowledge his indebtedness to Dr. C. M. Child for suggestions and criticisms.

Some of the questions that arose in the course of this investigation and furnished additional stimulus in the carrying-out of this research are: (1) Does the fate of the graft depend upon the degree of specialization, or does it depend upon the degree of physiological activity of the graft? (2) Does the active dominant region of the planarian body, such as the head or ganglionic region of the head (Child, 1929*b*), act as an organizer when transplanted into a relatively less active region of the body? (3) Is its effect species-specific? (4) On what factors does the fate of the graft depend? The work was undertaken in the hope of throwing additional light on the dynamics of histogenesis and of morphogenesis. Some of the more important results obtained thus far have already been briefly discussed in the preliminary paper (Santos, 1929).

Most of the earlier work on transplantation in the lower invertebrates has been chiefly concerned with other problems than those which are attacked in the present paper. Various investigators have grafted together pieces of hydroids, *Turbellaria*, or annelids in various positions. When such grafts are made in reversed polar orientation with respect to each other and one component is made much

smaller than the other, either at the time of grafting or by subsequent removal of a part, the polarity of the smaller component is often reversed. Such experiments, however, do not necessarily demonstrate induction by the larger component, for short isolated pieces of the species concerned often give rise to bipolar forms; and the reversal of polarity in the shorter components of the grafts in reversed position may represent merely the effect of the posterior or anterior cut surfaces in determining a new polarity, and inhibition of development at the anterior or posterior cut surface by union with the other component. Various other cases of alteration of polarity, or symmetry in consequence of transplantation, or of other operations which alter the positions and relations of parts to each other have been reported. The recent work of Spemann and his collaborators on the so-called "organizer" of the urodele embryo has greatly stimulated interest in the problem of the physiological relations of parts to each other in embryonic development and reconstitution; and "induction," that is, organizing or reorganizing action of a part or region on others, has been discovered or postulated in numerous cases. It is impossible to consider all such cases here; furthermore, recent general papers make such consideration unnecessary. For example, in the *Festschrift* for Spemann, Korschelt (1929) discusses morphogenetic induction, Goetsch (1929) the experimental influencing of regeneration, and Child (1929*b*) physiological dominance; and all of these papers give extensive bibliographies. It may perhaps be questioned whether certain cases which have been regarded as cases of induction, and particularly certain cases of reversal of polarity of anterior or distal regions by posterior or proximal regions, are really such; but the fact that certain regions are physiologically dominant over others and that such regions do exert an organizing or reorganizing action or induction in embryonic development and in reconstitution cannot be doubted. This action or influence appears both in the determination of the development of certain parts and in the prevention of development of others, but it is not necessary to assume that these two effects result from different physiological factors. The prevention or inhibition by a dominant region, or organizer of a certain sort of development in a certain region, undoubtedly results not from an actual inhibiting action

on the part of the dominant region but from the determination in the region concerned of some other line of development.

A few of the more striking cases of the morphogenetic effects of physiological dominance among the lower invertebrates are briefly mentioned. Browne (1909) found that even small pieces from the hypostome region of *Hydra*, transplanted to other regions of the host body, might determine a new apical region and axis. By using individuals of different colors as host and donor, she was able to show that the new individual determined by the graft developed in large part from the host body. Small pieces from other regions of the body had no such determining or inducing effect, but large pieces might persist and give rise to apical ends and new axes. Rand, Bovard, and Minnich (1926) have obtained similar results. The experiments of Goetsch (1922–26, 1929) on *Hydra* and other hydroids give further data concerning actual or postulated organizing action in these forms.

In the case of *Corymorpha*, Child (1929*a*) showed that even a very small piece of the stem grafted into a lateral incision in the stem of another individual would induce development of a new hydranth and stem, the graft forming only the apical portion of the hydranth.

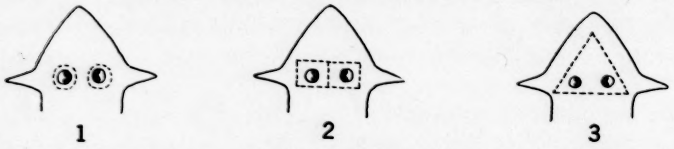
Most cases of induction in planarians which have been reported by earlier investigators consist in the prevention of development of a head or a posterior end when the relations of the region concerned to other parts, and particularly to a head region, are altered either by transplantation or by certain other operations. Various cases of this sort are mentioned by Korschelt (1929), Goetsch (1929), Rand and Ellis (1926), and Rand and Browne (1926); and Yiking Li (1928) also describes cases of this sort.

Previous investigators, however, have presented little evidence for actual organizing action or induction, that is, the determination of development of new parts by a transplanted part, e.g., a head. Some of the cases of heads developed from transplanted regenerating tissue described by Gebhardt (1926) suggest that the graft has organized or determined some slight outgrowth from the host body, but Gebhardt does not discuss this point. In some of the experiments of Goetsch (1929, pp. 264–66) the evidence is more conclusive.

In these experiments regenerating head regions were transplanted, and later the region of the host body about the transplant was isolated by section. The grafted head was often able to prevent entirely or in part the development of a head on the piece of the host body and also to reorganize the piece into an elongated structure, apparently a postcephalic region.

MATERIALS AND METHODS

Planaria dorotocephala Woodworth and the form commonly known as *P. maculata* Leidy were the animals used in this work. The latter species was obtained from the vicinity of New Haven and from Woods Hole through the kindness of Dr. L. G. Barth and Dr. J. W. Buchanan. The sizes of the worms used range from 10 to



FIGS. 1–3.—Planarian heads showing regions, shapes, and sizes of pieces used as ganglionic grafts.

22 mm. in length. A saturated aqueous solution of chloretone diluted with four parts of well water or a weak aqueous solution of chloretone (*ca.* M/100) was used in most cases as anesthetic. The donors were not anesthetized. In some cases neither the host nor the donor was anesthetized. In many of the earlier experiments circular grafts (Fig. 1) about $\frac{1}{2}$ mm. in diameter were cut by means of a capillary pipette and inserted into a hole in the host body with the same pipette. In other cases rectangular pieces representing either approximately a half or the whole of the ganglionic region (Fig. 2) were cut with a sharp scalpel and inserted into rectangular holes of as nearly as possible the same size. In the course of the experiments, however, it was found that triangular pieces were the most satisfactory. With pieces cut in the form of isosceles triangles, with the sides longer than the base, and implanted into a hole of approximately the same size and shape, definite orientation with respect to the polar axis of the donor and host is possible. In the case of the ganglionic region, equilateral triangular pieces were used, the presence of the eyes mak-

ing orientation possible (Fig. 3). Since the polarity of the graft and its effect on the polarity of the host are important features of the investigation, the advantages of the triangular pieces as grafts are obvious. The operated worms were kept in the dark, in moist chambers consisting of finger bowls and small watch glasses with very little well water, only enough to keep the worms moist, for about 12–24 hours immediately following the operation. After this length of time, those with successful grafts were examined under a binocular microscope, counted, recorded, and transferred into finger bowls with *ca.* 200 cc. fresh well water, covered with glass plates, and kept in the dark in the usual way. In some of the experiments the host was beheaded, to keep it relatively quiet and thus increase the percentage of "takes"; while in other cases the host was not beheaded. Both homoplastic transplantation and heteroplastic transplantation were performed, the latter between *Planaria dorotocephala* and *P. maculata*. In some of the experiments *P. maculata* was the host, while in others the host was *P. dorotocephala*. The region of the cephalic ganglia and various levels of the prepharyngeal, pharyngeal, and postpharyngeal regions were the different levels of the body that have been used as graft and, with the exception of the ganglionic region, as region of implantation in these experiments. The grafted piece was either transplanted without its axis being rotated or the axis of the transplanted piece was rotated 60°–180°. In some cases the piece was grafted with its dorsal surface toward the ventral surface of the host.

In general, the experimental animals which were able to feed were fed with beef liver two or three times a week. The pattern of the gut in the experimental animals has been determined either by examining the living animals under slight pressure with strong light and small diaphragm or by feeding the animals clotted blood. The parts of the gut which are filled from a particular pharynx in feeding and the movements of the gut contents serve to show the connections between the various parts of the reorganized guts.

The figures are semidiagrammatic outlines drawn in each case from the living animal to show the essential features of the results obtained. No attempt is made to indicate the actual difference in size of the animals in the figures. Some of the figures are drawn on a

somewhat larger scale than others in order to show clearly the essential points. Since no characteristic difference in results in relation to size of animals has been observed, this variation in scale of figures is regarded as justifiable. In the figures, the pharynx serves as a landmark and as evidence of induced reorganization. The reorganization of the gut in relation to the new pharynges is not shown in the figures but will be considered in a later paper in connection with the histological data.

GRAFTS OF THE GANGLIONIC REGION

Homoplastic grafts of the ganglionic region of Planaria dorotocephala.—The more important results obtained with homoplastic grafts of the ganglionic region of *Planaria dorotocephala* are summarized in Table I. The table does not include all the experiments performed with ganglionic grafts. In many of the earlier experiments with small circular or rectangular grafts (Figs. 1 and 2) complete resorption of the graft took place. Since the development of a head and the induction of an outgrowth by the graft were considered to be of greater interest than resorption, most of these experiments are not recorded in Table I. Some other experiments with the large triangular pieces (Fig. 3) in which only small numbers of animals were used are also omitted from Table I.

The table is in large part a record of the data obtained after the technique had been satisfactorily developed, and represents the entire range of results observed. For purposes of tabulation various series of experiments performed at different times in which implantation was made at approximately the same level of the body are combined under a single series number. The consecutive series numbers in the table represent successive levels of the body from the anterior prepharyngeal region posteriorly, as indicated in the third column. The fourth and fifth columns of the table give the numbers of successful grafts ("takes") and the numbers of unsuccessful cases ("non-takes") which were used as controls. The sixth column gives the cases in which at least some degree of development of a head from the graft and induction of an outgrowth occurred. Cases of resorption are not tabulated, since only two cases occurred (Series 5) among the experiments recorded in the table.

In many experiments the anterior end of the host was removed at a level somewhat anterior to the level of implantation after development of the graft head and induction of an outgrowth by the graft had taken place. Usually such removal is followed by development

TABLE I

HOMOPLASTIC GRAFTS OF GANGLIONIC REGION, *Planaria dorotocephala*, LENGTH 10–22 MM.

Series	Shape and Size of Graft	Level of Implantation	Number of "Takes"	Controls ("Non-Takes")	Induction of Outgrowth	Subsequent Removal of Anterior Part of Host	Head of Host Inhibited	Reversal of Polarity in Host
1....	Circular, one-half ganglion	Anterior prepharyngeal	7	23	7	7	5	.....
2....	Rectangular, or triangular, whole ganglion	Middle prepharyngeal	53	21	53	16	0	.....
3....	Rectangular, whole ganglion	Posterior prepharyngeal	38	11	38	17	5	.....
4....	Triangular, whole ganglion	Pharyngeal	31	17	31 (22 with 2 pharynges)	.....	.....	.....
5....	Rectangular or triangular	Middle postpharyngeal	52	84	50 (13 with second pharynx)	.....	.....	.....
6....	Triangular, whole ganglion	Middle and posterior postpharyngeal	125	75	125	125	59	113

of a new head, which may be more or less inhibited in its development. Removal of this reconstituted head may be followed by development of another head still more inhibited or by complete inhibition of head development at the anterior cut end of the host. After a third removal, head development is usually completely prevented. When the level of removal of the anterior end of the host is near the level of the graft, the effect of the graft in inhibiting

development of a new head is greater and fewer removals are necessary for complete inhibition of head development. Also, the more completely developed graft heads are in general more effective in inhibiting head development at more anterior levels than those which are less developed. And finally, ganglionic grafts in the postpharyngeal region are apparently more effective in inhibiting head development at levels of the host body anterior to the graft level than are such grafts in the prepharyngeal region.

In Table I the number of cases in which the anterior end of the host was removed once or more than once (number of removals not given in table) is given in the seventh column; and the number of cases in which development of a new head was inhibited or prevented, in the eighth column. The data show that such inhibition or prevention of head development is much more frequent in the case of grafts in the postpharyngeal than with prepharyngeal grafts. More extended consideration of this effect of the graft head in inhibiting head development at levels of the host body anterior to it is reserved for a later paper, in which data will be presented to show the gradual increase in this effect in particular cases.

When ganglionic grafts are implanted into the posterior part of the postpharyngeal region, a reversal of polarity in at least part of the host body is often induced by the graft. The cases of such reversal are recorded in the last column of Table I.

In general, it may be stated that the results of grafting pieces from the ganglionic region are always resorption when the pieces are below a certain size, or some degree of development from the graft of a head region which may be highly abnormal in form; and, except when implantation is very near the head of the host, induction of an outgrowth from the tissue of the host which represents more or less of the postcephalic region. In no case observed, however, does this induced body include regions posterior to the level of implantation.

Detailed consideration of all cases is, of course, impossible; but description of a number of cases selected from the data will serve to illustrate the character and range of variation of the results obtained with these homoplastic grafts of the ganglionic region. Figures 4 and 5 represent a case of implantation in the anterior prepharyngeal region just posterior to the head of the host (Series 1, Table I), the

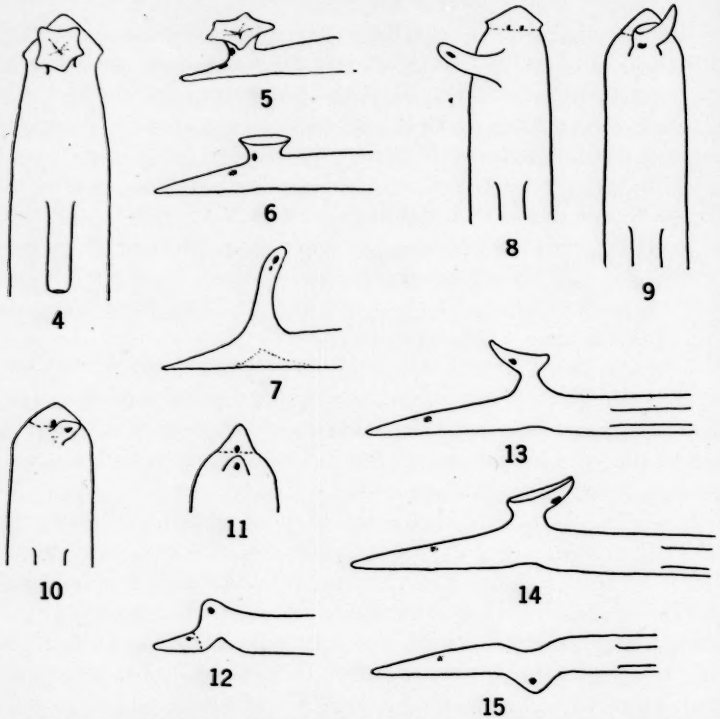
graft being implanted in reversed dorsoventral orientation. A highly abnormal head region developed with ventral surface consisting of the characteristic ventral epithelium uppermost. In Figure 5 the position of the eyes of the host and the single eye of the graft are indicated. The ventral surface of the reconstituted head region is concave, and the margin shows five pointed outgrowths. It was impossible to determine with certainty whether a particular one or each of these outgrowths represented an anterior tip. All five underwent contraction and extension simultaneously, and it seems possible that they all represent anterior tips and that this head region is multipolar to that extent.

Figure 6 shows a case somewhat similar to the preceding. The graft was implanted just posterior to the head in reversed dorsoventral orientation. The head region which develops from the graft is funnel-shaped with ventral epithelium on the inner, and dorsal epithelium on the outer, surface of the funnel.

In Figures 7 and 8 a case of implantation into the anterior prepharyngeal region (Series 1, Table I) with normal dorsoventral orientation is shown. Figure 7 is a lateral view; and Figure 8, a dorsal view with the outgrowth bent to one side. The outgrowth is cylindrical and covered with epithelium like that of the dorsal surface, but the eyespot indicates that its free end represents a head region. The ventral surface of the host at the level of the graft is concave in the dorsal direction. In this case the head of the host was removed after development of the outgrowth; but the development of a new head took place, as indicated in Figure 8.

Figure 9, another case of implantation with normal dorsoventral orientation in the anterior prepharyngeal region (Series 1, Table I), shows development of a highly abnormal head with a tapering outgrowth on one side. Here, also, the head of the host was removed after development of the graft, and a new head developed as indicated in the figure.

In Figure 10 another case from the anterior prepharyngeal region (Series 1, Table I) with normal dorsoventral orientation is shown, but the level of implantation is slightly more posterior than in the preceding cases. Here the head which develops from the graft is more nearly normal in form, and a short outgrowth posterior to it



FIGS. 4–15.—*Planaria dorotocephala*. Homoplastic ganglionic grafts in anterior and middle prepharyngeal regions.

Figs. 4–6, circular grafts in anterior prepharyngeal region, normal anteroposterior, reversed dorsoventral orientation. Union incomplete. Figs. 4 and 5, dorsal and lateral views of the same graft. Fig. 6, another similar graft.

Figs. 7 and 8, circular graft in anterior prepharyngeal region in normal orientation, union complete. Graft head covered with dorsal epithelium. Graft has induced postcephalic outgrowth. Fig. 7, lateral view; Fig. 8, dorsal view with graft bent to one side. Head of host removed after development of graft and development of new head not inhibited.

Fig. 9, triangular graft in anterior prepharyngeal region, in reversed anteroposterior and dorsoventral orientation, union incomplete. Head of host removed after development of graft, and new head partially inhibited.

Figs. 10–12, circular grafts in anterior prepharyngeal region in normal orientation. Union complete, graft head covered with dorsal epithelium. Head of host removed after development of graft, and new head partially inhibited. Fig. 11, dorsal view; Fig. 12, lateral view of same graft.

Figs. 13–15, triangular grafts in middle prepharyngeal region. Fig. 13, in normal anteroposterior, reversed dorsoventral orientation. Union incomplete. Fig. 14, in reversed anteroposterior and dorsoventral orientation. Union incomplete. Fig. 15, in normal orientation, union complete, graft head ventral, covered with ventral epithelium.

has arisen. The head of the host was removed after the graft developed; and the new head is somewhat inhibited, as is indicated by the median eyespot. Figures 11 and 12 are dorsal and lateral views of a similar case but with less outgrowth. Here, also, the head of the host was removed after development of the graft; and the new head is partially inhibited in development, as its form and the single median eye indicate (Fig. 11).

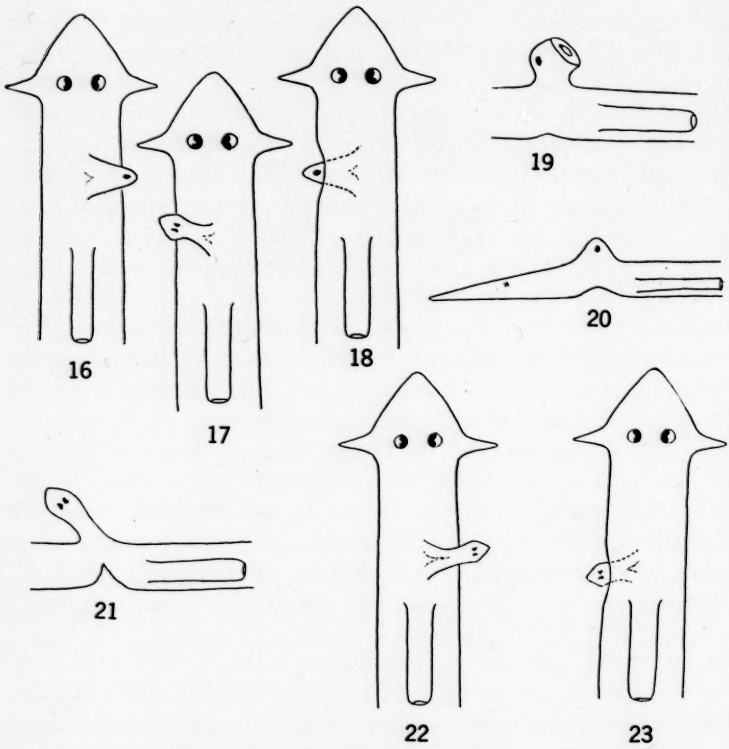
Figures 13 and 14 show two cases of implantation into the middle prepharyngeal region (Series 2, Table I) with reversed dorsoventral orientation and, in the case of Figure 14, with reversed anteroposterior orientation. In both cases the head develops from the graft with ventral epithelium on the concave upper surface; and in Figure 14, with its anterior end directed toward the posterior end of the host. In these cases the head of the host was not removed. In the case shown in Figure 15 the graft was implanted in normal position; but the head developed on the ventral side of the host, and the dorsal surface of the host became concave in the region of the graft. Here, also, the head of the host was not removed.

Figures 16, 17, and 18 show further cases of implantation in the middle prepharyngeal region (Series 2, Table I) with normal orientation of the graft. In Figures 16 and 17 the head and outgrowth develop on the dorsal surface of the host, and in Figure 18, on the ventral surface. In these cases the head of the host was not removed.

In Figures 19, 20, 21, 22, and 23 cases of implantation into the posterior part of the prepharyngeal region (Series 3, Table I) are shown. In the case of Figure 19 the graft was in reversed dorsoventral orientation; and, as in other cases with similar orientation described above, the head develops with ventral epithelium on the concave upper surface. Figures 20–22 show cases with normal orientation of graft and development of head and outgrowth on the dorsal surface of the host. In the case of Figure 23 the graft was implanted in reversed dorsoventral orientation, and the head and outgrowth developed on the ventral surface of the host. The head of the host was not removed in any of these cases.

Grafts of the ganglionic region into the pharyngeal region (Series 4, Table I) also developed a prepharyngeal region from the host body. In most cases, also, development of a second pharynx in the host body was induced. One of these cases is shown in Figure 24.

Here the pharyngeal region of the host was completely removed and replaced by the graft. The host has developed a new pharynx, and the graft has induced development of a cylindrical prepharyngeal



FIGS. 16–23.—*Planaria dorotocephala*. Triangular, homoplastic grafts in middle and posterior prepharyngeal region.

Figs. 16–18, grafts in middle prepharyngeal region. Dorsal views with outgrowth bent to one side, normal orientation. Fig. 16, union complete, graft head covered with dorsal epithelium. Fig. 17, union incomplete, head normal. Fig. 18, union complete, graft head ventral, covered with ventral epithelium.

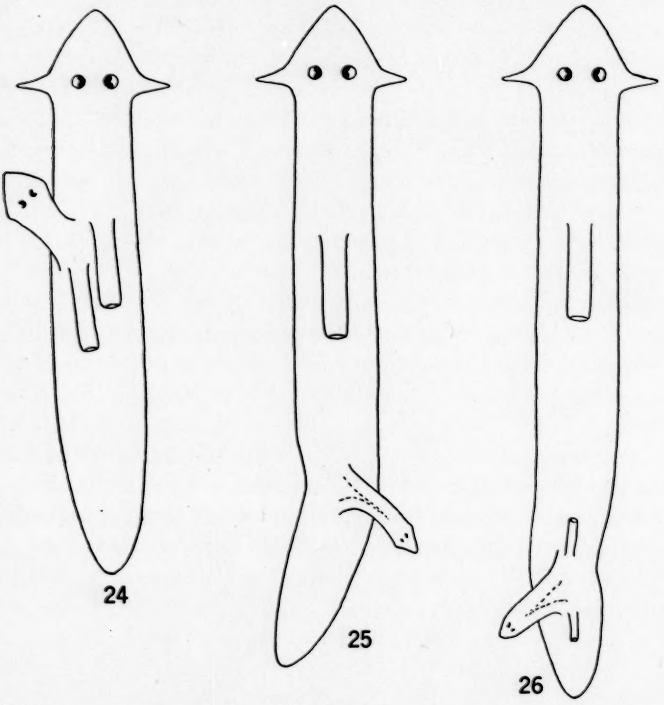
Figs. 19–23, grafts in posterior prepharyngeal region. Fig. 19, normal anteroposterior, reversed dorsoventral orientation, union incomplete. Fig. 20, normal orientation, union complete, graft head covered with dorsal epithelium. Figs. 21 and 22, normal orientation, union incomplete, lateral and dorsal views of same graft. Fig. 23, normal orientation, union incomplete, graft head and outgrowth ventral.

region from the host body and the development of a secondary pharynx slightly posterior to that of the host. Reorganization of the gut in relation to the induced pharynx has also taken place but is not shown in the figure.

The general results obtained with homoplastic grafts of the ganglionic region of *P. dorotocephala* into the postpharyngeal region are given in Series 5 and 6 of Table I. Series 5 consists of cases of ganglionic grafts into the middle postpharyngeal region without subsequent removal of the anterior end of the host; and Series 6, of cases of ganglionic grafts into the middle and posterior postpharyngeal region with subsequent removal of the anterior end of the host at some level anterior to the graft. In Series 5 the usual result is some degree of head development and induction of a postcephalic outgrowth from the body of the host (Fig. 25). In these cases the induced outgrowth unquestionably represents a new polarity, determined by the graft; but there is no indication of reversal of polarity in other parts of the host body, such as occurs in Series 6. In general, such reversal of polarity occurs much more frequently when the anterior end of the host is removed after the graft and outgrowth have developed; but in a few cases, which are not included in the tabulated data, reversal of polarity did occur without removal of the anterior end of the host. A case of this sort is shown in Figure 26. Here the graft has induced a postcephalic outgrowth and the development of two pharynges, one anterior to the graft and in reversed position, the other posterior to the graft and in normal position. The gut (not shown in the figure) is reorganized in relation to the two pharynges. In this case the graft has induced reorganization in the host body for some distance anterior and posterior from the level of implantation, while in the more anterior levels the original organization of the host body remains unchanged.

As Series 6, Table I, shows, numerous cases of reversal of polarity in the postpharyngeal region of the host body have resulted from implantation of the ganglionic region into postpharyngeal levels, with subsequent removal of the anterior end of the host. Figures 27 and 28 show cases of this sort. In both of these cases the head which has developed from the graft has become the dominant region and functionally the anterior end.

In some cases of homoplastic ganglionic grafts in the extreme posterior postpharyngeal region of *P. dorotocephala* development of a head and induction of a postcephalic outgrowth occurred, but no



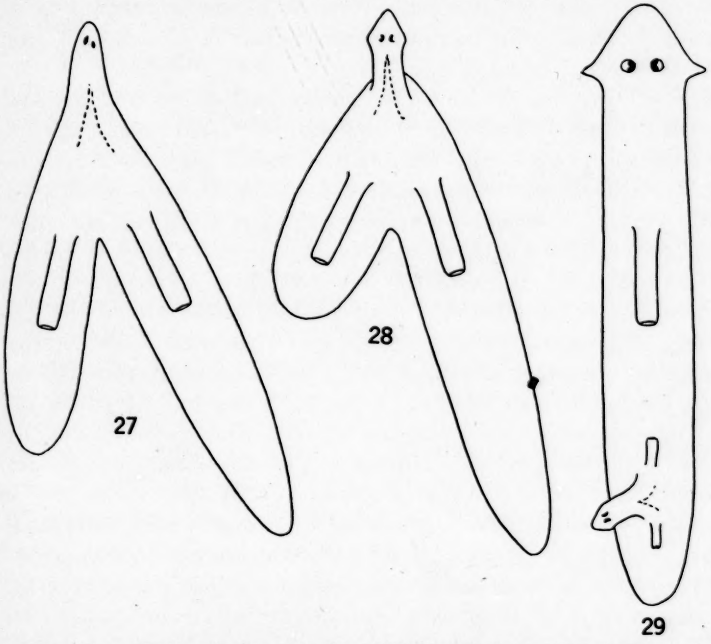
FIGS. 24–26.—*Planaria dorotocephala*. Triangular, homoplastic ganglionic grafts in pharyngeal and postpharyngeal regions.

Fig. 24, graft in pharyngeal region, union incomplete, head normal. Graft has induced postcephalic outgrowth from host body and new pharynx in host body.

Fig. 25, graft in postpharyngeal region in reversed anteroposterior, normal dorsoventral orientation, union incomplete, graft head with both dorsal and ventral epithelium, induced postcephalic outgrowth with dorsal epithelium external, ventral epithelium in invagination.

Fig. 26, graft in postpharyngeal region in reversed anteroposterior, normal dorsoventral orientation, union incomplete, graft head with dorsal and ventral epithelium, induced postcephalic outgrowth with dorsal epithelium external, ventral epithelium in invagination. The graft head has also induced development of two pharynges in host body, one in normal, one in reversed polar orientation.

visible reversal of polarity anterior to the graft level took place—that is, no pharynx developed during the period of observation.



FIGS. 27–29.—Triangular homoplastic ganglionic grafts in postpharyngeal region in normal orientation. Figs. 27 and 28, *Planaria dorotocephala*. Fig. 29, *P. maculata*.

Fig. 27, union complete, head and postcephalic outgrowth covered with dorsal epithelium. Graft head has induced development of two pharynges in opposite polar orientation in host body. Anterior end of host removed posterior to pharynx after development of graft and development of new head prevented by graft and polarity of anterior portion of host body reversed (left side of figure).

Fig. 28, a case similar to Fig. 27 except that union is incomplete and graft head is normal.

Fig. 29, graft in posterior postpharyngeal region, union incomplete, head with dorsal and ventral epithelium; induced postcephalic outgrowth with dorsal epithelium external. The graft head has induced postcephalic outgrowth from the host body and also development of two pharynges in opposite orientation in host body. Anterior end of host was not removed.

These cases, however, were among the earlier experiments and were kept under observation for only about four weeks. Later experi-

ments have shown that reversal of polarity by the graft often does not become evident in so short a time. It is probable, therefore, that in at least some of these cases reversal of polarity anterior to the graft would have taken place if the animals had been kept for a longer time.

It will be observed from the figures that the length of the postcephalic outgrowth and the effect of the graft in inducing reorganization in the host body differ in different cases at the same level of the body. This difference is most clearly evident in grafts in the postpharyngeal region. In some of the cases described and figured above, the grafts into the postpharyngeal region develop a head and induce a postcephalic outgrowth but do not induce development of a pharynx or pharynges and reorganization of the gut in the host body (Fig. 25, also Santos, 1929, Fig. 22). In other cases development of one or two pharynges and corresponding reorganization of the gut in the host body are induced by the graft (Figs. 26–28). These differences are perhaps due in part to differences in size of the graft, in its degree of union with the host tissues, or in its physiological condition in relation to that of the host. It is also possible that in some cases the grafted animals were not kept under observation long enough for complete development of the effect of the graft. The induced development of new pharynges in the host body occurs rather slowly, in most cases requiring several weeks. In all cases the grafted animals remained under observation for at least a month, and often for three or four months. Many of them were finally fixed for histological examination, and it is possible that in some cases further-induced reorganization in the host body might have occurred if the animals had been kept for a longer time. In most of the later experiments the animals were kept until it appeared certain that no further development would occur.

Certain characteristics of the heads which develop from the grafts and of the induced postcephalic outgrowths require some further consideration. The form of the graft head ranges from normal with cephalic lobes through various abnormal forms to bluntly pointed or rounded head regions more or less conical in form with a single eye. The degree of healing of the graft into the host body is an important factor in determining these differences in head form. For

example, some portion of the cut surface of the graft may fail to unite with the host tissues. Under these conditions this part of the graft heals like any other cut surface, and the dorsal and ventral epithelium of the graft unite with each other. In such cases the head which develops shows the characteristic dorsoventral difference in pigmentation and appearance of dorsal and ventral epithelium. Heads of this sort usually develop cephalic lobes and may be completely normal (Figs. 4, 5, 6, 9, 10, 13, 14, 17, 19, 22, 23, 24, 26, 28).

When the piece is grafted with normal dorsoventral orientation and the graft unites completely with the tissue of the host, dorsal epithelium of the graft with dorsal epithelium of the host and ventral epithelium with ventral, the graft head is rounded or bluntly pointed anteriorly and more or less conical in form, and is completely covered with the epithelium characteristic of the surface from which it grows out. A part of this epithelium belongs to the graft; a part, to the host. If it develops on the dorsal surface of the host, it is covered with dorsal epithelium (Figs. 7, 8, 10, 11, 12, 16, 27); if from the ventral surface, with ventral epithelium (Figs. 15, 18). So far as can be determined, no transformation of dorsal epithelium into ventral or of ventral epithelium into dorsal takes place. Such heads do not develop cephalic lobes and usually possess only a single eye, which may be elongated or otherwise abnormal in form. These facts suggest that union of dorsal and ventral epithelium of the graft is one of the necessary conditions for the development of the cephalic lobes.

When the graft is implanted with reversed dorsoventral orientation, the union with the host tissues is often less complete than with normal dorsoventral orientation, and the graft head possesses both dorsal and ventral epithelium but reversed in position with respect to the host (Figs. 4, 5, 6, 13, 14, 19).

In short, it appears from the facts that grafts of the ganglionic region retain their dorsoventrality but that in the cases of complete union of graft and host, that is, of dorsal with dorsal and ventral with ventral, the graft head does not develop both dorsal and ventral epithelium but is covered with the epithelium of the body surface from which it grows out.

As regards persistence of the polarity of the graft, the facts are not

so clear. In cases with reversed dorsoventral and anteroposterior orientation, the original polarity persists (Fig. 14); but in many other cases, particularly those in which the graft head is merely a rounded outgrowth from the dorsal or ventral surface of the host, it is impossible to determine whether or not the original polarity persists in the graft, for there is no indication of it in the form of the head. In the case of Figure 4 the five pointed regions may be anterior head regions; and if they are, they indicate a multipolar condition at least as regards the head tip.

The postcephalic outgrowth determined by the graft differs in certain respects from a normal postcephalic region. It is very commonly more or less cylindrical instead of flattened. If it develops from the dorsal surface of the host, it is covered on all sides with dorsal epithelium (Figs. 7, 8, 10, 13, 14, 16, 17, 19, 20, 21, 22, 24, 25, 26, 27, 28); and if it develops from the ventral surface, it is covered with ventral epithelium (Figs. 15, 18, 23). In all cases when the union between the graft and host is complete, and in many cases when it is not complete, a depression or invagination appears on the side of the host body opposite to that on which the outgrowth develops and at the same level as the outgrowth. As elongation of the outgrowth takes place, this depression may extend as a tubular invagination partly or completely through the body of the host and sometimes a greater or less distance into the induced outgrowth, in some cases all the way to the graft head. This characteristic of the induced outgrowth ranges, then, from a mere depression or concavity on the surface opposite the graft to an elongated tubular invagination extending the whole length of the outgrowth. In the figures of cases in which it is present, it is shown either as a depression in the surface (Figs. 12–15, 19–21) or by dotted lines when indicated as inside the induced outgrowth (Figs. 7, 8, 10, 16, 18, 20, 21, 22, 25, 26, 27, 28). When the outgrowth develops from the dorsal surface of the host, this invagination is lined with ventral epithelium (Figs. 7, 8, 10, 12, 16, 18, 20, 21, 22, 23, 25, 26, 27, 28), which apparently, however, becomes somewhat modified in the deeper regions of the more elongated invaginations. When the outgrowth develops from the ventral surface of the host, the invagination is lined with dorsal epithelium (Figs. 15, 23).

In short, the induced postcephalic outgrowth may be tubular in form with dorsal epithelium on the outer and ventral epithelium on the inner surface, or vice versa. It cannot then be regarded as a normal postcephalic region, but the fact that in the grafts into postpharyngeal levels new pharynges and postpharyngeal regions are determined in relation to it indicates that it is physiologically a postcephalic region. Likewise, the fact that the graft head may dominate the host body and control its motor activity through the induced outgrowth points to the conclusion that the outgrowth functions as a postcephalic region. Further histological data concerning the graft head and the induced outgrowth will appear in a later paper.

Homoplastic grafts of the ganglionic region of Planaria maculata.—The number of homoplastic grafts of the ganglionic region of *Planaria maculata* which have been made is not as large as in the case of *P. dorotocephala*. Since the results obtained in the two species are essentially similar, tabulation and extended description of cases is regarded as unnecessary. In *P. maculata* the development of a head from the graft and the induction of a postcephalic outgrowth from the host body occur as in *P. dorotocephala*, and the various head forms and their relation to the degree of union between graft and host are similar in the two species. The development of a depression or tubular invagination on the surface of the host body opposite the graft is also of frequent occurrence in *P. maculata* as in *P. dorotocephala*. Two cases of homoplastic grafts of the ganglionic region into the postpharyngeal region of *P. maculata* are described and figured in the earlier paper (Santos, 1929, Figs. 25, 27), and a third similar case is shown in Figure 29 of the present paper. In all three of these cases the graft developed a head, induced a postcephalic outgrowth from the host body, and also determined the development of two pharynges and corresponding reorganization of the gut in the host body. One of the pharynges is anterior to the level of the graft and in reversed position with relation to the original polar axis of the host. The other is posterior to the graft level and in normal position with relation to the host axis (Fig. 29). In this case, as in similar cases in *P. dorotocephala*, the graft head possesses both dorsal and ventral epithelium and is normal in form with cephalic lobes while the induced postcephalic outgrowth is entirely covered on its surface

with dorsal epithelium but shows an invagination lined with ventral epithelium from the ventral surface of the host.

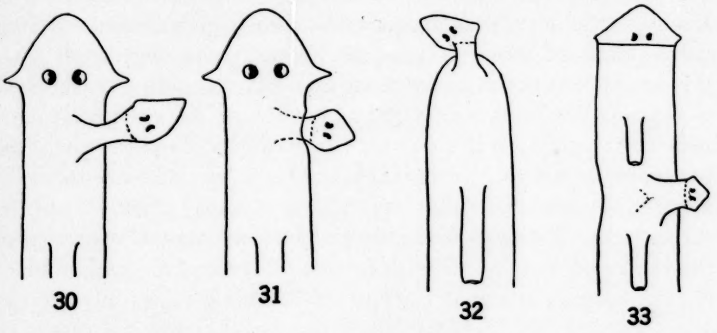
Heteroplastic grafts of the ganglionic region between Planaria dorotocephala and P. maculata.—More than one hundred heteroplastic grafts of the ganglionic region between *Planaria dorotocephala* and *P. maculata* have been made, both species serving both as donor and as host. The results are similar to those obtained with the homoplastic grafts in the two species. The point of chief importance with respect to the heteroplastic grafts is that the ganglionic region of one species is able to induce a postcephalic outgrowth from the body of the other species and, when implanted into the postpharyngeal region, to induce development of one or more pharynges and reorganization of the gut in the body of the host species. In other words, the physiological effect of the graft on the body of the host is not species-specific.

The data obtained, however, indicate one important difference between the two species. In general, grafts of the ganglionic region of *P. dorotocephala* seem to be more effective in inducing postcephalic outgrowth and, in the postpharyngeal region, the development of new pharynges and reorganization of the gut in the body of *P. maculata* than the ganglionic grafts of *P. maculata* into *P. dorotocephala*. With the ganglionic grafts of *P. dorotocephala* the induction of development in the body of *P. maculata* occurs more frequently and more rapidly than in the reverse case. Similarly, with small grafts representing a part of the ganglionic region resorption occurs less frequently when the graft is from *P. dorotocephala* than when it is from *P. maculata*.

A number of cases of heteroplastic ganglionic grafts between these two species have already been figured and briefly described in an earlier paper (Santos, 1929). In that paper, Figures 9, 13–15, 21, 23, 24, 26, 28, show cases of heteroplastic ganglionic grafts between *P. dorotocephala* and *P. maculata*. A few of these cases are more fully described in the present paper together with some cases not included in the earlier paper.

Since the results obtained with heteroplastic ganglionic grafts are similar to those described above for homoplastic grafts, and since figures and descriptions of cases of heteroplastic grafting have been

given in the earlier paper, only a few characteristic cases need be described here. In Figure 30 a case of implantation of the ganglionic region of *P. dorotocephala* into the anterior postpharyngeal region of *P. maculata* is shown. The *P. dorotocephala* head possesses both dorsal and ventral epithelium of *P. dorotocephala* and is normal in form. The induced postcephalic outgrowth has developed from the dorsal surface of the host and is carried with the dorsal



FIGS. 30–33.—Triangular heteroplastic ganglionic grafts in prepharyngeal and pharyngeal regions.

Figs. 30 and 31, grafts of *Planaria dorotocephala* in *P. maculata* in normal orientation, union incomplete, head with dorsal and ventral epithelium of donor. Fig. 30, graft head dorsal on host, induced postcephalic outgrowth covered with dorsal epithelium of host. Fig. 31, graft head ventral on host; induced postcephalic outgrowth covered with ventral epithelium of host.

Figs. 32 and 33, grafts of *P. maculata* in *P. dorotocephala*. Fig. 32, graft in anterior prepharyngeal region, normal orientation, union incomplete, graft head with dorsal and ventral epithelium of donor, induced postcephalic outgrowth covered with dorsal epithelium of host. Anterior end of host removed after development of graft, and graft head has prevented development of a new head on host after the second removal. Fig. 33, graft in pharyngeal region, reversed anteroposterior, normal dorsoventral orientation, union incomplete, graft head with dorsal and ventral epithelium of donor, induced postcephalic outgrowth covered with dorsal epithelium of host. The graft head has induced development of a pharynx posterior to the graft level and the host has reconstituted a pharynx anterior to the graft level. Anterior end of host removed after development of graft and new normal head developed.

epithelium characteristic of *P. maculata*. In this case there is no invagination on the ventral surface of the host body. Figure 31 shows a case of *P. dorotocephala* graft in *P. maculata* at the same body level

as Figure 30. In this case, however, the head and the induced outgrowth developed on the ventral side of the host body. The head is normal in form and possesses both the dorsal and ventral epithelium of *P. dorotocephala*, while the induced outgrowth is covered with the ventral epithelium of *P. maculata*. No invagination from the dorsal surface of the host has developed. In Figure 32 (Santos, 1929, Fig. 9), which shows a ganglionic graft from *P. maculata* implanted in the prepharyngeal region of *P. dorotocephala* at the same level as Figures 30 and 31, the anterior end of the host was removed after development of the graft head, and a new head developed. After this second removal at a level just anterior to the graft, development of the host head was completely inhibited, and the graft head functioned as the head of the animal. Other cases of implantation of the ganglionic region of *P. dorotocephala* into *P. maculata* are shown in Figures 13–15 of the earlier paper (Santos, 1929). Figure 13 of the earlier paper is similar to Figure 30 described above. Figure 14 of the earlier paper is a case with reversed dorsoventral orientation of the graft and is similar to the homoplastic graft shown in Figure 13 of the present paper. Figure 15 of the earlier paper is a case with reversed dorsoventral and anteroposterior orientation of the graft similar to the homoplastic graft in Figure 14 of the present paper. Other cases of successful heteroplastic ganglionic grafts in the prepharyngeal region differ from the homoplastic grafts only in the fact that the graft head possesses the epithelium and, when fully developed, the form characteristic of the donor species, while the induced outgrowth develops from the host species and its outer surface is covered with epithelium characteristic of the surface of the host species. Also, in case an invagination from the opposite surface of the host body occurs, this is lined with epithelium characteristic of that surface of the host species.

In Figure 33 a graft of the ganglionic region of *P. maculata* into the pharyngeal region of *P. dorotocephala* is shown. The graft head is a normal *P. maculata* head, and the postcephalic outgrowth from the dorsal surface of the host is covered with dorsal *P. dorotocephala* epithelium. In this case the pharyngeal region and the head of the host were removed. A new normal head has developed at the anterior cut end of the host body, and a new pharynx, in normal posi-

tion anterior to the graft level. The development of this pharynx is undoubtedly determined by the new dominant head region at the anterior end of the host body, not by the graft head. Posterior to the level of the graft another pharynx, evidently determined by the graft head, has developed. Here the *P. maculata* graft head has had no visible effect on levels anterior to it but has determined reorganization at more posterior levels of the *P. dorotocephala* body.

The heteroplastic ganglionic grafts at postpharyngeal levels are of greater interest than the prepharyngeal because, as in the homoplastic grafts, they induce more extensive reorganization in the host body than grafts at more anterior levels. In Table II numerical

TABLE II

HETEROPLASTIC GRAFTS OF GANGLIONIC REGION BETWEEN *Planaria dorotocephala* AND *Planaria maculata;* LENGTH, 10–22 MM.

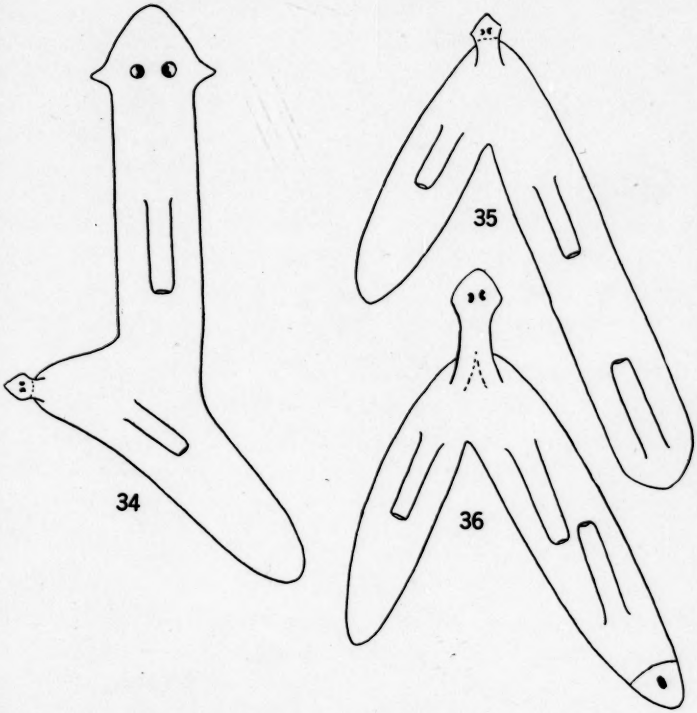
All grafts were triangular and included approximately the whole ganglionic region, and all were implanted in the postpharyngeal region of the host.

Series and Species of Host	Number of "Takes"	Controls ("Non-Takes")	Induction of Outgrowth	Anterior End of Host Removed	Reconstitution of New Head Inhibited	Reversal of Polarity
1. *Planaria dorotocephala*	24	33	24	.....	.....	.....
2. *P. dorotocephala*	3	17	3	3 (fission posterior to pharynx)	3	.....
3. *P. dorotocephala*.....	3	1	3	3	2	.....
4. *P. maculata*	10	2	10	4	.....	4
5. *P. maculata*	5	5	5	5	5	5
6. *P. maculata*	19	3	19	.....	19	19

data are given for the most successful series of such grafts. Series in which most of the grafts failed to unite with the host tissue or were resorbed are not included in the table. In Series 1, 2, 3, *P. dorotocephala* is host and *P. maculata* donor; in Series 4–6, *P. maculata* is host and *P. dorotocephala* donor. The table shows that in all "takes" induction of an outgrowth from the host body occurred. In the cases in which *P. dorotocephala* was host (Series 1, 2, and 3) there was, however, no reversal of polarity in the host body. In the three cases of Series 2 and in the two cases of Series 3 the anterior end of the host was removed posterior to the pharynx after the graft head and outgrowth developed, and the graft head prevented development of a

new head from the anterior cut surface of the host body. In Series 4–6, with *P. maculata* as host, the anterior end of the host was removed in all cases after the graft head and induced outgrowth developed. As in the case of similar homoplastic grafts, a new head, either normal or more or less inhibited in form, developed on the anterior end of the host after this first removal. This head was then removed, and in some cases a head developed again but more inhibited in form than the first, while in other cases no head developed. In cases in which the second head did develop, it was also removed; and after this second removal of the reconstituted head, further head development at the anterior end of the host might be completely prevented and the anterior end might be transformed into a posterior end. Here, as in the homoplastic grafts, the dominance of the graft head evidently extends gradually in the anterior direction to the anterior cut end of the host body; and, even though it does not completely inhibit reconstitution of a head at first, it may do so after a second or third removal of the head and may finally reorganize that portion of the host body anterior to the graft level as a posterior end. The nearer the level of removal of the anterior end of the host to the level of the graft and the more nearly normal the graft head, the sooner does complete inhibition of development of a new host head, and reorganization of the anterior portion of the host body into a posterior end, occur. The last two columns of Table II give only the final results as regards inhibition of development of the host head and reversal of polarity in the host body anterior to the graft level.

A few characteristic cases are selected for description. In Figure 34 (Santos, 1929, Fig. 21) a case of *P. dorotocephala* graft into the anterior postpharyngeal region of *P. maculata* is shown. The graft head is normal, and the induced postcephalic outgrowth from the host body is short; but the host body is drawn out laterally in the region of the graft head, into a broad blunt extension. To what extent this change in form of the host body represents growth and to what extent deformation is uncertain. It developed gradually, apparently in relation to the mechanical tension exerted by the graft head in its efforts to advance. Reorganization in the case of Figure 34 has been induced only posterior to the level of the graft, and there



FIGS. 34–35.—Triangular, heteroplastic ganglionic grafts of *Planaria dorotocephala* in postpharyngeal region of *P. maculata*. Union incomplete, graft heads with dorsal and ventral epithelium of donor, induced postcephalic outgrowth covered with dorsal epithelium of host.

Fig. 34, graft in normal orientation, induction of short postcephalic outgrowth, lateral extension of host body, and new pharynx posterior to graft level.

Fig. 35, graft in reversed anteroposterior, normal dorsoventral orientation. Graft has induced postcephalic outgrowth and two new pharynges, one posterior to graft level in normal polar orientation (left), the other anterior to graft level in reversed polar orientation (right). After development of graft, anterior end of host was removed posterior to host pharynx. A new head and pharynx developed. Head was removed, new head again developed; but after removal of this, development of head was prevented by graft (lower right in figure).

FIG. 36.—Graft in reversed anteroposterior, normal dorsoventral orientation. Induction as in Fig. 35. Anterior end of host removed twice; second reconstituted head partially inhibited by graft (lower right in figure). *Planaria maculata* triangular, ganglionic graft in *P. dorotocephala* body.

is no reversal of polarity at more anterior levels. The graft was made at about the level of fission, so that the region anterior to it is a part of the first zooid and under the dominance of the host head. This fact probably accounts for the absence of induced reorganization anterior to the graft levels. Both the homoplastic ganglionic grafts in the postpharyngeal region already described and similar heteroplastic grafts to be described below show that at more posterior postpharyngeal levels (that is, well within the posterior zooid region of the host which is more or less physiologically isolated from the dominance of the head) reorganization anterior as well as posterior to the graft level may occur.

In the case of Figure 35, a ganglionic graft from *P. dorotocephala* near the middle of the postpharyngeal region of *P. maculata*, the graft has developed a *P. dorotocephala* head and induced postcephalic outgrowth covered with dorsal epithelium from the *P. maculata* body. The host body has become bent upon itself at the level of the graft apparently in consequence of locomotion with the graft head in advance. The anterior end of the host body is on the right side of the figure. Development of a pharynx and corresponding reorganization of the gut has taken place both posterior and anterior to the graft level, representing in the latter case a reversal of polarity with pharynx in reversed position. In this case the head of the host was removed when the graft was made, and a new head developed. The anterior end was again removed after the graft head had developed and reorganization in the host body had taken place. After this removal no head developed at the anterior end of the host, although the persistence of the host pharynx indicates that polarity has not been completely reversed in this region. Apparently, the graft head has inhibited head development at the anterior end of the host body even though the level of the cut surface is at a considerable distance anterior to the graft.

Figure 36 shows a case very similar to Figure 35, but with a *P. maculata* graft in a *P. dorotocephala* body. The graft head has induced reorganization in the host body anterior and posterior to its level. The anterior end of the host was cut off in the pharyngeal region, and a new normal head and pharynx developed. This head was removed; and the second reconstituted head, as shown in Figure

36, was teratomorphic, i.e., partially inhibited. Further removal was not made in this case.

In the case shown in Figure 37 (Santos, 1929, Fig. 26) the *P. maculata* graft is implanted at a more posterior level of the postpharyngeal region of *P. dorotocephala;* and reorganization and pharynx development occur both posterior and anterior to the graft level, even though the head of the host is present. The level of the graft in this case is well within the posterior zooid region, and the presence of the host head does not prevent reversal of polarity anterior to the graft level.

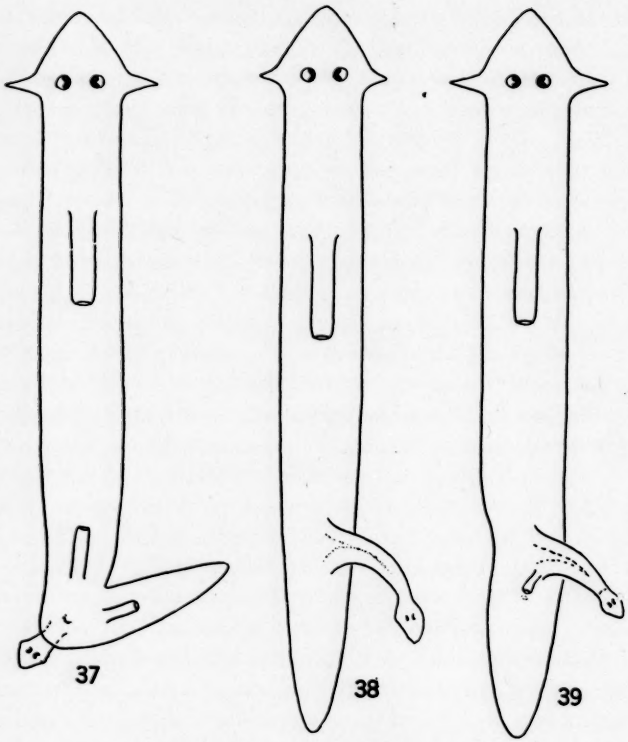
In Figure 38 the *P. dorotocephala* graft developed a head and induced development of a postcephalic outgrowth from the *P. maculata* body but did not determine further visible reorganization within the period of observation, which in this case was 34 days. Further reorganization might have occurred later. In Figure 39 (Santos, 1929, Fig. 29), a *P. maculata* graft in *P. dorotocephala*, a graft head and induced host outgrowth appear, and a small pharynx has developed just posterior to the level of the induced outgrowth.

In the case shown in Figure 40 (Santos, 1929, Fig. 28) the *P. maculata* graft was implanted in the extreme posterior postpharyngeal region of *P. dorotocephala*. The graft head induced development of a postcephalic outgrowth and reversal of polarity in the host body for a considerable distance anterior to its level, as indicated by the development of a pharynx in reversed position. The dotted outline at the right in Figure 40 indicates the position assumed by the graft head and the region of reversed polarity during forward locomotion.

In the figures of heteroplastic grafts of the ganglionic region given in the present paper the boundary between the epithelium of the graft head and that of the induced postcephalic outgrowth is approximately indicated, but the difference in pigmentation characteristic of the two species which is clearly evident in the living animals is not shown. Figures showing this difference in pigmentation will be given in connection with the histological data.

GRAFTS FROM POSTCEPHALIC REGIONS

The data concerning grafts of pieces from postcephalic regions are less complete than those concerning ganglionic grafts. It became



FIGS. 37–39.—Triangular, heteroplastic ganglionic grafts in postpharyngeal region in normal orientation, union incomplete, graft heads with dorsal and ventral epithelium of donor.

Fig. 37, graft of *Planaria maculata* in *P. dorotocephala*. Graft head on ventral side of host. Induced postcephalic outgrowth covered with ventral epithelium of host. Graft has induced development of two pharynges in host body, one posterior to level of graft in normal orientation, one anterior to level of graft in reversed polar orientation.

Fig. 38, graft of *P. dorotocephala* in *P. maculata*. Induced postcephalic outgrowth covered externally with dorsal epithelium of host; ventral invagination lined with ventral epithelium.

Fig. 39, graft of *P. maculata* in *P. dorotocephala*. Induced postcephalic outgrowth covered externally with dorsal epithelium of host; ventral invagination lined with ventral epithelium. Graft has induced development of one small pharynx just posterior to graft level.

evident early in the course of the work that the ganglionic grafts were of greater interest in many respects than the postcephalic grafts; consequently it seemed desirable to obtain, first of all, fairly complete data concerning them. A large number of grafts from prepharyngeal, pharyngeal, and postpharyngeal levels of the donors into prepharyngeal and pharyngeal regions of the host have been made; but the results of grafting postcephalic pieces into postpharyngeal regions have not yet been determined. Up to the present, also, most of the grafts of postcephalic pieces have been homoplastic, and in *P. dorotocephala*. Further experiments along these lines are planned for the near future.

Data concerning postcephalic grafts are presented here chiefly for the purpose of showing some of the differences between these and the ganglionic grafts.

Homoplastic grafts of pieces from the prepharyngeal region.—In all except a few of the earlier experiments the pieces grafted were in the form of isosceles triangles. In some cases the base of the triangular piece grafted represented almost the whole width of the body and was either anterior or posterior (Fig. 41). In other cases the triangular piece was taken from one side of the body, the base repre-

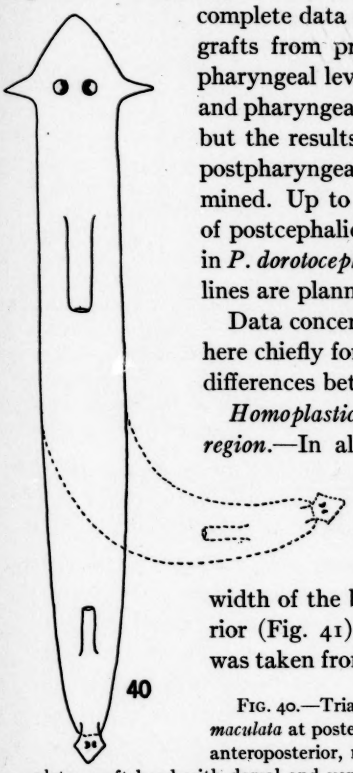
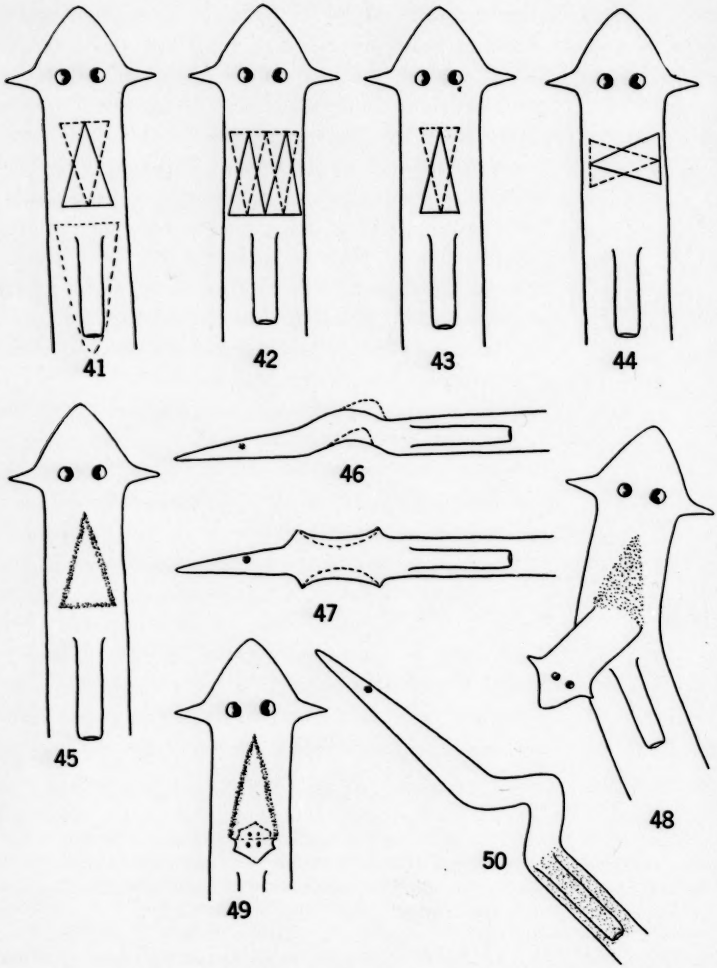


FIG. 40.—Triangular heteroplastic ganglionic graft of *Planaria maculata* at posterior end of body of *P. dorotocephala* in reversed anteroposterior, normal dorsoventral orientation. Union incomplete; graft head with dorsal and ventral epithelium of donor; induced postcephalic outgrowth covered with dorsal epithelium of host. Graft has induced development of new pharynx in reversed polar orientation in body of host.

senting slightly less than one-half the body width and being either anterior or posterior (Fig. 42), and was implanted in the median region (Fig. 43). In still other cases the grafts were cut, as in Figure 44, and grafted with the base of the triangle either anterior or posterior on the host body (Fig. 41). The experiments do not cover all the possible orientations of graft in relation to the host, but



FIGS. 41–50.—Homoplastic grafts of triangular pieces from prepharyngeal region of *Planaria dorotocephala*.

Figs. 41–44, to show the region of body, shape, size, orientations and levels of implantation of triangular pieces used as grafts from prepharyngeal region. Pieces with bases posterior (Figs. 41–43) or toward right (Fig. 44) drawn in solid lines; pieces with bases anterior (Figs. 41–43) or toward left (Fig. 44) drawn in broken lines. The triangu-

lar outlines in Fig. 41 also indicate approximately the level of implantation of grafts into the prepharyngeal and the pharyngeal regions.

Fig. 45, dorsal view of graft of prepharyngeal piece into prepharyngeal region in normal orientation. Union complete.

Fig. 46, lateral sectional view of prepharyngeal piece into prepharyngeal region in reversed anteroposterior, normal dorsoventral orientation. Union complete. Graft is incorporated with slight growth of new tissue along lines of union.

Fig. 47, lateral sectional view of prepharyngeal piece into prepharyngeal region in normal anteroposterior, reversed dorsoventral orientation. Union complete.

Fig. 48, dorsal view of graft of prepharyngeal piece into prepharyngeal region in reversed anteroposterior, normal dorsoventral orientation. Union incomplete. Graft head and postcephalic outgrowth both developed from graft and both normal, possessing dorsal and ventral epithelium. Graft has migrated posteriorly and original region of implantation is filled in with new tissue, indicated by shading.

Fig. 49, dorsal view of graft of prepharyngeal piece into prepharyngeal region in reversed anteroposterior, normal dorsoventral orientation. Union incomplete. Graft has developed a normal head from its anterior end, and the host has developed a normal head on its cut surface facing anteriorly at posterior end of implantation opening.

Fig. 50, lateral sectional view of graft of prepharyngeal piece into pharyngeal region. The graft is in normal orientation. The graft has migrated anteriorly, part of the original region of implantation is filled in with new tissue (indicated in the figure by shading), and new pharynx has developed in it.

grafts of prepharyngeal pieces into the same level of the host have been made in normal anteroposterior with normal or reversed dorsoventral orientation, in reversed anteroposterior with normal dorsoventral orientation; also, grafts of prepharyngeal pieces into the pharyngeal region with reversed anteroposterior and normal dorsoventral orientation have been made—more than three hundred cases of complete or partial union of graft and host in all; and in many cases subsequent operations were performed, chiefly for the purpose of determining the effect of orientation of the graft in the host body. It is impossible to present all the various results at this time, but some of the more important are briefly described.

Considering first the grafts of prepharyngeal pieces into approximately the same body level of the host as that from which the graft was taken, it is found that such grafts in normal orientation usually unite completely with the host body and are incorporated in it (Fig. 45). They may give rise at first to a slight dorsal elevation and ventral invagination never greater than that indicated by the unbroken lines in Figure 46, which gradually disappears and is often very slight or absent from the beginning.

Grafts with reversed anteroposterior and normal dorsoventral orientation which unite completely with the host body show a more pronounced and more persistent dorsal elevation (Fig. 46, dotted lines) than those in normal orientation, and a gradual change in position of the elevation in the posterior direction often occurs with the development of new tissue in the host body at the original level of implantation and anterior to the graft. In cases with normal anteroposterior and reversed dorsoventral orientation and with complete union, elevation occurs both dorsally and ventrally about the margins of the graft while the middle region is concave both dorsally and ventrally (Fig. 47). The marginal elevation probably indicates that more growth occurs along the line of union than with normal dorsoventral orientation (Fig. 46).

Grafts in reversed anteroposterior and either normal or reversed dorsoventral orientation show incomplete union more frequently than those with normal orientation. In most such cases observed, it was the anterior end of the graft which failed to unite, and a head developed from it. Figures 48 and 49 show cases of this sort. In the case of Figure 48 a normal head develops on the original anterior end of the graft, now directed posteriorly; and the tissue of the graft is gradually drawn out, apparently by the attachment and mechanical tension exerted by the head, and forms a normal postcephalic region. The original position of the graft is largely occupied by new tissue, indicated in Figure 48 by shading. In the case of Figure 49 a head, directed posteriorly, develops from the original anterior end of the graft; and another, directed anteriorly and lying beneath the graft head, from the anterior cut surface of the hole in the host body. One case has been observed in which the posterior region of a grafted piece failed to unite and gave rise to a posterior end which developed on the ventral side of the host.

Some fifty grafts of prepharyngeal pieces into the pharyngeal region with complete or partial union have been made. In these cases a triangular piece including the whole pharyngeal region is removed from the host, and the triangular graft inserted in the hole thus made. Figure 50 indicates the usual result in cases of complete union in normal orientation. The region of the graft at first becomes dorsally elevated to a greater extent than in grafts at prepharyngeal

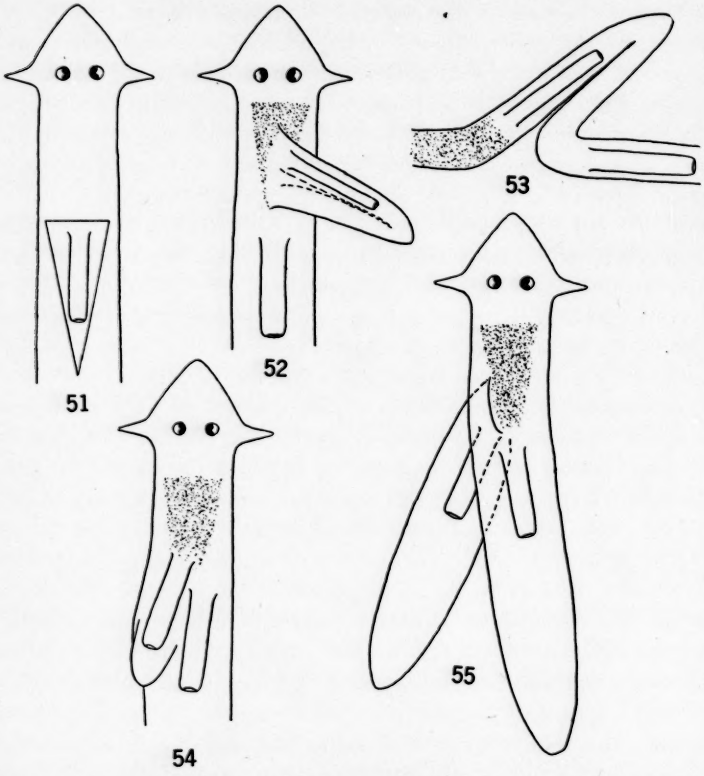
levels (Fig. 46), and a corresponding concavity appears on the ventral surface; but this distortion gradually disappears. Also, the graft appears to migrate in the anterior direction, and an area of new tissue develops posterior to it at the original level of implantation. In this area of new tissue posterior to the graft a new pharynx develops. This change in position of the graft evidently results from the difference in physiological condition at different levels of the body. In these cases a piece is implanted at a level posterior to its original level and gradually changes its position toward its original level. As will appear below, migrations either in the anterior or posterior direction occur in other cases. Discussion is postponed until these further cases have been described.

Homoplastic grafts of pieces from the pharyngeal region.—Pieces including the whole pharyngeal region with the base of the triangle anterior (Fig. 51) were transplanted to the prepharyngeal region with normal orientation. When healing is complete, such grafts give rise to an elongated dorsal elevation with a tubular invagination from the ventral surface (Figs. 52 and 53). The pharynx of the graft lies on the anterior side of this elevation, and the mouth opens into the ventral invagination near the apex of the elevation (Figs. 52 and 53). This position of the mouth, which was originally close to the posterior end of the graft, indicates that the posterior side of the dorsal elevation originates from the host tissue. This graft gradually migrates posteriorly toward the pharyngeal region of the host, that is, toward the body level corresponding to that from which the graft was taken. In Figure 54 another case is shown. Here the graft implanted at a level entirely anterior to the pharynx of the host has migrated so far posteriorly that the graft pharynx lies almost at the same level as the host pharynx but somewhat dorsal to it.

The case shown in Figure 55 is also a graft of the pharyngeal region into the prepharyngeal region but with incomplete union between graft and host and development of a posterior end containing the graft pharynx. This posterior end extends beneath a part of the lateral region of the host body but is entirely free from this part, so that an opening through the host body between the lateral strip and the new posterior end exists, as indicated in Figure 55.

Grafts of the pharyngeal region into the pharyngeal region have

been made only in reversed anteroposterior and normal dorsoneutral orientation. These grafts were cut as indicated in Figure 51 and



FIGS. 51–55.—Homoplastic grafts of triangular pieces from pharyngeal into prepharyngeal region in normal orientation in *Planaria dorotocephala*.

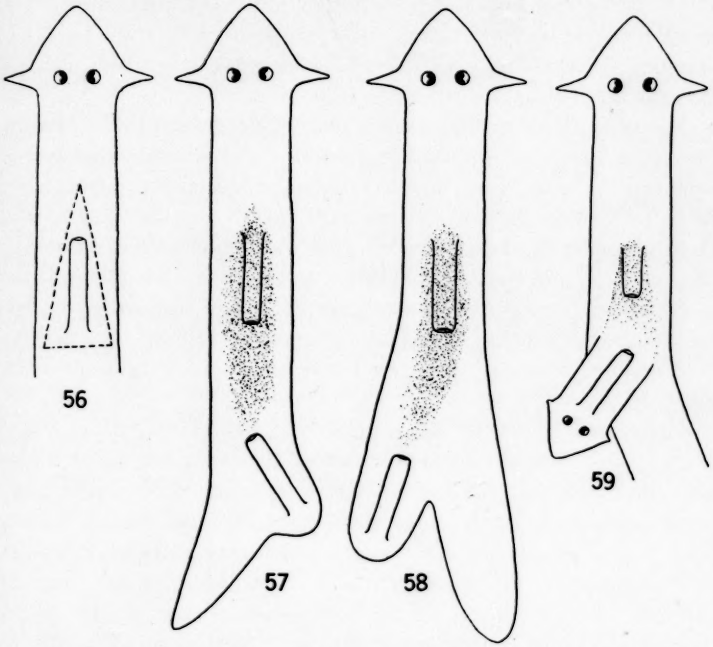
Fig. 51, the triangular outline indicates the piece used as graft.

Figs. 52 and 53, dorsal and lateral sectional views of same graft. Union complete. Graft has given rise to a dorsal elevation with corresponding ventral invagination and migrates posteriorly, the original region of implantation being filled in with new tissue, indicated by shading.

Fig. 54, dorsal view of another case in which the direction of migration is posteriolateral, the original region of implantation being filled in with new tissue.

Fig. 55, dorsal view of another case of incomplete union. The graft has given rise to a new posterior end and has migrated posteriorly, the original region of implantation being filled in with new tissue.

implanted in the position indicated in Figure 56. Such grafts also develop a dorsal elevation and ventral invagination; but instead of remaining in the pharyngeal region in which they were implanted, they migrate posteriorly, and sooner or later approach one side or the other of the body so that the original dorsal elevation finally



FIGS. 56–59.—Homoplastic grafts of triangular pieces from the pharyngeal region into the pharyngeal region in reversed anteroposterior, normal dorsoventral orientation.

Fig. 56, the triangular outline shows the position of the graft after implantation.

Figs. 57 and 58, union complete. Grafts have migrated posteriorly; the original region of implantation is filled in with new tissue, and a new pharynx has developed in it.

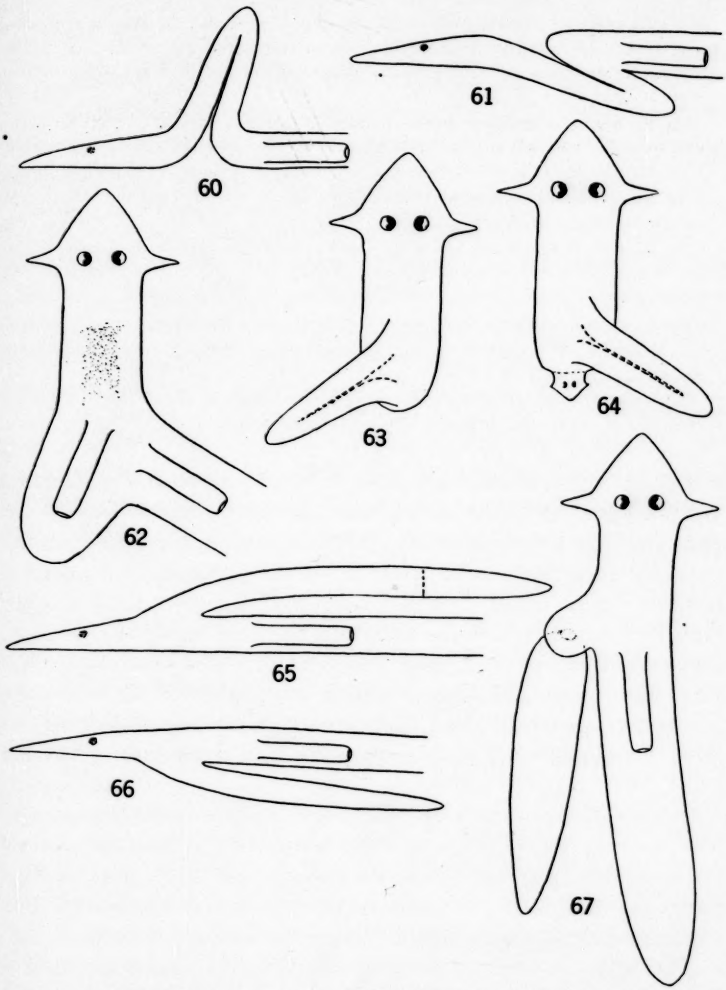
Fig. 59, union incomplete. The graft has developed a normal head at its anterior end and a postcephalic region, and has migrated posteriorly, the original region of implantation being filled in with new tissue, indicated by shading.

extends more or less posteriorly from a lateral margin of the body (Figs. 57 and 58). During this migration the ventral invagination gradually disappears; and when the condition of Figures 57 and 58

is attained, the dorsal side of the graft extension is covered with dorsal epithelium, the ventral side with ventral epithelium. The graft pharynx remains functional and is able to ingest food. In these cases the fact that the graft in reversed anteroposterior orientation does not remain at the body level corresponding to that from which it was taken, but migrates posteriorly, that is, with its anterior end in advance, is of particular interest since this migration results in bringing the graft to a body level far from the level from which it was taken.

Figure 59 shows another case of pharyngeal graft into the pharyngeal region with reversed anteroposterior and normal dorsoventral orientation. In this case, however, the anterior end of the graft failed to unite with the host tissue and gave rise to a normal head and a short prepharyngeal region developed from the graft tissue between the new head and the graft pharynx. In this case, also, the graft has migrated posteriorly and to a large extent out of the host body, and represents the prepharyngeal and pharyngeal regions of a new individual. In Figure 59 the new tissue anterior to the graft is indicated by shading.

Homoplastic grafts of pieces from the postpharyngeal region.—Sixty grafts from the posterior part of the postpharyngeal region into the prepharyngeal region with normal orientation gave complete union with the host. In such cases the graft region usually gives rise to an elongated elevation, which may be either dorsal or ventral, and a corresponding ventral or dorsal invagination (Figs. 60 and 61). In such cases it becomes impossible sooner or later to determine the exact boundaries of the graft, but apparently little or no migration occurs. In some cases, however, the dorsal elevation gradually migrates posteriorly and toward one side of the body, forming a lateroposterior extension (Fig. 62). Here the graft region develops a new pharynx, the ventral invagination disappears, and the final result is an apparently normal pharyngeal and postpharyngeal region formed in part from the graft and in part from the lateral region of the host body. This may become the chief posterior end of the individual and may undergo fission. In a number of these postpharyngeal grafts into the prepharyngeal region the posterior portion of the host body was cut off just posterior to the



FIGS. 60–67.—Homoplastic grafts of triangular pieces from the posterior postpharyngeal region into the prepharyngeal region. *Planaria dorotocephala.*

Figs. 60 and 61, lateral sectional views showing dorsal or ventral elevation and corresponding ventral or dorsal invagination in cases of complete union in normal orientation.

Fig. 62, normal orientation, union complete. Graft at first formed a dorsal eleva-

tion. This migrated posteriorly and laterally; and as it reached the margin of the host body, ventral invagination disappeared, reorganization into a pharyngeal and postpharyngeal region took place, and gradual elongation followed in stages later than that shown in figure.

Fig. 63, normal orientation, union complete. After development of graft elevation, posterior end of host was removed just posterior to level of graft, and no regeneration occurred at the posterior cut surface.

Fig. 64, graft and subsequent operation as in Fig. 63. Posterior cut end of host body developed a normal head.

Fig. 65, normal orientation, union incomplete. Graft gave rise on dorsal side of host body to a posterior end which elongated and underwent fission at level indicated by broken line.

Fig. 66, normal anteroposterior, reversed dorsoventral orientation, union incomplete. Graft developed into a posterior region in reversed dorsoventral orientation with respect to host.

Fig. 67, normal orientation, union incomplete. Graft developed into a posterior region attached to ventral surface of extension of the host body.

elevation. In such cases there was no outgrowth of new tissue from the posterior cut surface of the host body to form a new posterior end; but the graft elevation gradually took the position of the posterior end, the ventral invagination disappeared, and a normal posterior region resulted, with development of a new pharynx either anterior to or in the anterior portion of the graft tissue. Meanwhile, gradual elongation of the whole body took place. Figure 63 represents an early stage in this process of reorganization. It shows the absence of regeneration of a posterior end by the host body, but the graft elevation has not yet undergone reorganization into a posterior end.

In three cases in which the posterior end of the host was removed just posterior to the graft, a normal head developed from the posterior cut surface (Fig. 64). The conditions concerned in this development of a head from the posterior cut surface are not known, but the possibility is suggested that the graft has cut off nervous connection between the head of the host and the posterior cut surface and so brought about a sufficient degree of physiological isolation of this region to permit a head to develop.

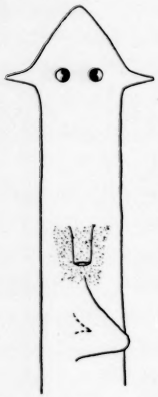
In all cases observed of incomplete union of postpharyngeal grafts into the prepharyngeal region, the posterior region of the graft has failed to unite and has given rise to a new posterior region, which

develops on either the dorsal or the ventral surface of the host and which may become as long as the host body (Figs. 65–67). In the case of Figure 67 the graft united with the host only at its anterior end, and the posterior outgrowth was somewhat to the left of the median line of the host. Here the graft finally came to lie entirely to the left of the host body and was connected with the host only by a slender peduncle; and the host body developed an extension originally ventral but gradually becoming lateral, apparently in consequence of the mechanical tension exerted by the outgrowth. These outgrowths function like normal posterior regions, and in the case of Figure 65 fission occurred at the level indicated by the dotted line in the figure; but unfortunately it was not determined whether they possessed pharynges. In these cases the elongation apparently results from growth of the graft, not from induction or reorganization in host tissues. This is indicated by the fact that in the case of Figure 65 the graft outgrowth arises from the dorsal surface of the host body but possesses, throughout its whole length, normal ventral epithelium on its ventral surface, which faces the dorsal surface of the host. Similarly in the case of Figure 66, a graft in reversed dorsoventral orientation, the graft outgrowth possesses normal dorsal epithelium on its lower surface throughout its length although it arises from the ventral surface of the host. The outgrowths induced from the host body are covered with the epithelium of the surface from which they arise.

Some seventy grafts of postpharyngeal pieces into the pharyngeal region were made with normal orientation. In cases of complete union the graft gave rise to more or less dorsal elevation and migrated posteriorly, while new tissue and a new pharynx developed anterior to it in the region where it was implanted. Figure 68 shows a case of this sort, the dorsal elevation being shown as bent over to one side and the area of new tissue indicated by shading. This direction of migration and the position of the new pharynx in relation to the graft are exactly the opposite of those observed in the grafts of prepharyngeal pieces into the pharyngeal region (see p. 38).

In seventeen cases grafts from the middle postpharyngeal region into the pharyngeal region with normal anteroposterior and reversed dorsoventral orientation united with the host body and developed

as shown in Figures 69 and 70. As the figures indicate, the grafts developed an elongated growth on the dorsal surface of the host and migrated from the pharyngeal region, where they were implanted, almost to the posterior end of the host body. The growth represents a small planarian body, ventral surface uppermost, and attached by part of its dorsal surface to the dorsal surface of the host. In a few cases a normal head was present (Fig. 69); a few others possessed more or less inhibited heads; but the majority were headless (Fig. 70).



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FIGS. 68–70.—Homoplastic grafts of triangular pieces from postpharyngeal into pharyngeal region. *Planaria dorotocephala.*

Fig. 68, union complete. Graft elevation has migrated posteriorly and original region of implantation is filled in with new tissue (indicated by shading) and has developed a new pharynx.

Figs. 69 and 70, normal anteroposterior, reversed dorsoventral orientation. Grafts have migrated posteriorly and have developed into small planarian bodies. Fig. 69, with normal head; Fig. 70, headless. The original region of implantation and the path of migration are filled in with new tissue (indicated by shading) in which a new pharynx has developed in each case.

DISCUSSION

Determination of reorganization in the host body by the ganglionic grafts.—The most important result of the experiments thus far is the demonstration of the ability of the ganglionic graft to induce more or less extensive reorganization in the host body. Size of the

graft, degree of union with the host, and apparently also the physiological condition of the graft in relation to that of the host are factors concerned in determining whether or not the graft induces reorganization. The smaller the graft, the more frequently it is resorbed without inducing reorganization. Also, when some part of the graft fails to unite with the host tissue, its own dorsal and ventral epithelium unite with each other, and the head which develops possesses both dorsal and ventral epithelium and is more frequently normal than the head which develops when union is complete. When the epithelia of the graft unite completely with those of the host, the head which develops is not normal, being without cephalic lobes, usually monophthalmic, and covered with the epithelium characteristic of that surface of the body from which it develops. Even such heads, however, induce more or less reorganization in the host body; but this inducing action is less rapid and often less extensive than that of a normal head. It appears probable that such differences in the reorganizing ability of the graft head are due to differences in its physiological condition.

The heteroplastic transplantations between *Planaria dorotocephala* and *P. maculata* show that the action of the ganglionic graft in inducing reorganization is not species-specific. Ganglionic grafts from each of the two species induce reorganization in the other species, although the data indicate at present that the ganglionic graft from *P. dorotocephala* is somewhat more effective in *P. maculata* than vice versa. It is difficult, however, to obtain really demonstrative data concerning this point because of the differences between the two species. *Planaria dorotocephala* usually becomes larger than *P. maculata* and has a larger head. If we use animals of the same size as donor and host, the individuals of *P. dorotocephala* are probably physiologically younger than those of *P. maculata;* and this difference may play a part in determining the efficiency of the graft. If, on the other hand, animals of different size are used, the grafts from the two species are either of different size or do not represent corresponding body regions. If the *P. dorotocephala* graft is actually more effective in *P. maculata* than vice versa, it is not clear whether the difference is due to differences in the grafts or in the reactivity of the host tissues of the graft.

The reorganization induced in the host body by the graft head appears in two different forms. That part of the prepharyngeal region which develops in relation to the graft head develops as an outgrowth from the host body; while in cases in which a pharynx and postpharyngeal region is reorganized by the graft, they develop in the host body without outgrowth (Figs. 24, 26–29, 34–37, 39, 40). In no case observed has the postcephalic outgrowth represented more than the prepharyngeal region, and it usually does not represent the whole prepharyngeal region. At any given level the maximum outgrowth is never longer than that portion of the host body anterior to the level of implantation, and it may be shorter according to the degree of development of the graft head.

The induced postcephalic outgrowth is not normal in its structural characteristics even when the h[illegible] is normal. It is usually more or less cylindrical in transverse [illegible]on and is covered with the epithelium characteristic of that [illegible]face of the host body from which it arises. In many cases it contains an elongated, more or [illegible]ubular invagination from the opposite body surface, and this invagination is lined with the epithelium characteristic of that surface. In the case of heteroplastic grafts the epithelia of the induced postcephalic outgrowth are those of the host, not the donor species. The question may perhaps be raised whether this induced outgrowth really represents a postcephalic region. As regards this question, perhaps the most conclusive evidence that this outgrowth is physiologically a postcephalic region is that even when it is tubular in form reorganization of the host body with development of pharyngeal and postpharyngeal regions may be induced through it. Apparently the induced outgrowth is a more or less radially symmetrical postcephalic region, its radial symmetry resulting from the fact that it develops from either the dorsal or the ventral surface of the host body. Evidently the dorsoventrality of the normal graft head does not induce transformation of dorsal into ventral or ventral into dorsal epithelium in the host. Moreover, in many cases, the graft head itself is essentially radially symmetrical. Certainly in such cases no factor is present which could determine dorsoventrality in the induced outgrowth.

When a ganglionic graft is implanted into the prepharyngeal re-

gion of the host, the induced postcephalic outgrowth never includes pharyngeal or postpharyngeal regions, and apparently represents at most only that region of the body corresponding to the region of the host body anterior to the level of implantation. When reorganization of the host by the graft has taken place to this level, it evidently does not continue further because the more posterior levels of the body are already represented in the host; and since, if the effect of the graft extends to these regions, it could determine only the organs that are already present, the graft does not determine reorganization in them. When ganglionic grafts are implanted in the postpharyngeal region, the situation is very different, as will appear.

With the ganglionic grafts in the prepharyngeal region, the visible reorganization induced by the graft does not extend in the host body beyond the base of the postcephalic outgrowth; but when such grafts are implanted in the pharyngeal or postpharyngeal region, more or less reorganization may occur in the host posterior to, and often in the case of postpharyngeal grafts both posterior and anterior, to the postcephalic outgrowth. This reorganization consists in the development of a new pharynx and a corresponding reorganization of the gut. When it takes place anterior to the graft level, it involves complete reversal of polarity in the part of the host body concerned; while still farther anterior, the original polarity may still persist. In such cases three polar axes are present in the host body: in the more anterior region a part of the original axis; then a new polarity reversed in direction which is determined by the graft; and posterior to the graft level a polarity which coincides in direction with the original but is nevertheless a new polarity determined by the graft, since the development of a new pharynx and extensive reorganization are involved. In Figures 26–29 and 34–37, for example, and in other cases not figured, these three polarities exist. In some cases when the anterior end of the host is removed, the polarity is completely reversed at levels anterior to the graft, and head formation is inhibited at the original anterior end or it may be transformed into a posterior end (Figs. 27, 28, 35).

The fact is to be emphasized that neither the postcephalic outgrowth nor the reorganization of pharyngeal and postpharyngeal region in the host body represent a regeneration in any strict sense.

The development of the postcephalic outgrowth is much slower than reorganization, and no "new" or embryonic tissue is visible in it. Histological study of its development is not yet complete. Still less is the reorganization of pharyngeal and postpharyngeal regions a regeneration. It takes place very slowly without essential change of form of the body, though it undoubtedly involves cell division and growth in the development of the new pharynx and the reorganization of the gut. It is a familiar fact that in the reconstitution of postpharyngeal pieces the development of the new pharynx and postpharyngeal region represents the same sort of reorganization that is induced by the ganglionic graft. In both cases a head region is brought into or near to the postpharyngeal region and induces its transformation. That the factors involved in such reorganization must differ very considerably from those concerned in the regeneration of new tissue from a cut surface seems evident.

The results obtained with these ganglionic grafts, both homoplastic and heteroplastic, demonstrate very clearly the physiological dominance of the ganglionic region in development. Evidently the ganglionic region is the primary factor in determining and localizing the various postcephalic parts. It is even able to determine and localize such parts in a direction opposed to a polarity already existing. In short, it is an "organizer" in the sense in which Spemann and his co-workers have used the word.

Moreover, the ganglionic graft is not only an organizer, but it may prevent the development of a head at an anterior cut surface of the host body a considerable distance anterior to the level of implantation. This inhibition of the host head is probably simply one aspect of the organizing effect of the graft, that is, it inhibits or prevents head formation because it determines conditions in the parts concerned which favor or determine development of other parts than a head. In some cases this effect is merely sufficient to inhibit head formation so that a teratophthalmic, a teratomorphic, an anophthalmic, or even no head at all develops instead of a normal head. The ganglionic grafts show all degrees of this effect from mere partial inhibition of head development to complete transformation of an anterior into a posterior end (Figs. 27, 28, 35, 36).

When the ganglionic graft is implanted at or near the level of the

postpharyngeal region at which fission usually occurs, fission at that level is prevented in all cases observed, and the region of fission is reorganized by the graft (Figs. 27, 28). In some cases in which the graft was implanted posterior to the level at which fission usually occurs, fission at the usual level took place soon after implantation, but the graft head prevented the development of a head at the anterior end of the fission piece and functioned as the head of the piece.

In many cases of ganglionic grafts in the postpharyngeal region it was found that the effect of the graft head on a cut surface of the host body anterior to its level developed only very gradually. Often a normal head developed after the first removal of the anterior end of the host, a somewhat inhibited head after the second removal, and complete prevention of head development after the third removal. In some cases the effect develops more rapidly, in others less rapidly, in still others it remains slight or is apparently absent and there is no reversal of polarity. Such differences are apparently correlated to some extent with differences in degree of development of the graft head. In general, the more nearly normal the graft head, the greater its effect in preventing head formation at more anterior levels of the host body and in reversing polarity. The gradual development of this dominance and determining action of the graft head suggests that it may be associated with a gradual reorganization of the nervous system progressing from the graft head to a greater or less distance from the host body.

The question may be raised as to the factors concerned in determining that the induced prepharyngeal region develops as an outgrowth from the host body while the induced pharyngeal and postpharyngeal regions develop through reorganization within the host body, that is, without outgrowth. A complete answer to this question cannot be given, but attention may be called to certain points. First, the development of the induced postcephalic outgrowth occurs more rapidly than the reorganization in the postpharyngeal region. Apparently the ganglionic graft induces more or less outgrowth before the reorganization of more posterior levels has time to occur.

Second, the figures show that the length of this outgrowth is variable, particularly in postpharyngeal implantation, even when the level of implantation is approximately the same; and also, that the

scale of organization of the new axis or axes differs widely in different cases. The induced pharynx or pharynges may arise at a considerable distance from the base of the postcephalic outgrowth or almost at its base. Apparently a relation exists between the effect of the graft in inducing outgrowth from the host body and in inducing reorganization in the host body posterior or anterior to the outgrowth. In other words, the postcephalic outgrowth may represent physiologically a larger fraction of the new polar axis in some cases than in others, and the reorganization in the host body posterior or anterior to the level of implantation apparently represents the levels of the new axis which are not represented in the postcephalic outgrowth.

Third, the rapidity with which the postcephalic outgrowth develops and the scale of organization in the new axis are probably determined by the degree to which the graft head influences the host, and they in turn determine how much of the new axis is represented by the outgrowth.

Fourth, the dominance of the graft head in postpharyngeal implantation does not necessarily extend to the posterior end of the host body. Most of the ganglionic grafts in the postpharyngeal region were implanted at or near the level at which fission would normally occur. In such cases the graft always prevents the occurrence of fission. In the normal individual the anterior end of the second zooid is only a short distance posterior to the mouth, and the same is probably true in individuals like Figures 27, 28, 35, 36, and 37. Thus far no attempt has been made to determine whether the posterior end of the host body is to some extent physiologically isolated from the dominance of the graft head. The reorganization does not afford any evidence on this point, since this region remains a postpharyngeal region even after the induced reorganization; and physiological isolation cannot be expected to alter its structure since the structure of the posterior zooid region is not appreciably altered in the normal individual.

Thus far no case of fission in a transformed anterior cut end of the host body after removal of more anterior portions (Figs. 27, 28, and 35) has been observed; and such cases are not to be expected, for, if the dominance of the graft head is sufficient to prevent formation of

a head and to transform the anterior end into a posterior end, it is evident that the degree of physiological isolation in such a region cannot be sufficient to permit fission unless considerable increase in length or a decrease in dominance occurs.

In short, the results of ganglionic grafts in the postpharyngeal region indicate that the graft head determines a new polar organization which is represented in part by the postcephalic outgrowth, in part by reorganization in the host body; that the new axis thus determined may differ greatly in length, that is, in scale of organization in different cases; and that the portions of the axis represented respectively by the postcephalic outgrowth and the reorganization in the host body also differ in different cases with the degree of dominance of the graft head over the body of the host.

The grafts of postcephalic regions.—The data presented concerning the grafts of postcephalic regions serve chiefly to indicate something of the variety of results attained with such grafts. The figures show that under certain conditions such grafts may determine a very considerable dorsal or ventral elevation or outgrowth even when union is complete. Very few heteroplastic grafts of postcephalic regions have been made as yet, and it is impossible in most cases of homoplastic grafts to determine how much of such outgrowths develops from the graft itself and how much is induced from the host body. In the case of grafts of the pharyngeal region into prepharyngeal levels (Figs. 52 and 53), the position of the mouth suggests that the posterior side of the outgrowth has developed from the host body; but in other cases there is no landmark.

Unquestionably these elevations or outgrowths represent more or less reorganization, which may involve to a greater or less extent regions of the host body adjoining the graft; but what the elevation or outgrowth represents physiologically and what conditions determine its development the data at hand do not show. It must be left for future investigation to determine whether these grafts of postcephalic regions are able, in certain cases, to determine some degree of definite reorganization in the host body beyond that associated with the union of the graft and host tissues.

When union is incomplete, the postcephalic graft may develop a head and a postcephalic outgrowth or a posterior end. Undoubtedly

the heads in such cases possess the same ability to reorganize the host body as do the heads arising from ganglionic grafts; but in these cases the first effect of the head is on the graft itself, and this may be induced by the head to develop into a postcephalic outgrowth. Such reorganization is similar to that which occurs in the reconstitution of an isolated piece. Unfortunately, no case of heads developing from postcephalic grafts in consequence of incomplete union have been observed in postpharyngeal implantation. Consequently, it is not known whether such heads will determine reorganization and reversal of polarity in the host body as do the heads which develop from ganglionic grafts. There is, however, every reason to believe that they will have the same effect.

The migrations of the postcephalic grafts present an interesting problem. In most cases the postcephalic graft implanted into a different body level with normal orientation appears to migrate toward the level from which it was taken, and new tissue develops in the region of implantation. Orientation, however, is a factor in determining the direction of migration. Grafts of the pharyngeal region implanted into the same region in reversed anteroposterior and normal dorsoventral orientation migrate in the posterior direction (Figs. 57–59) instead of remaining in the pharyngeal region, and new tissue and a new pharynx develop in the pharyngeal region of the host. Apparently the graft is not simply forced out of the position of implantation by the development there of new tissue, but is itself a factor in the migration. Further study of these migrations is planned in the hope of throwing some light on the factors concerned.

The polarity and dorsoventrality of the graft.—Apparently the dorsoventrality of the graft persists in all cases in which it is not resorbed, and in many cases it is evident that the polarity also persists. But in those cases among the ganglionic grafts in which the head region is a rounded, bluntly pointed structure with a single eye, it is impossible to determine whether the original polarity of the graft persists or not. In cases of postcephalic grafts in reversed anteroposterior and normal dorsoventral orientation it is often possible to determine by later operation whether or not the original polarity persists. Such operations show that in certain cases the original polarity of the graft is obliterated and a new polarity is determined,

but these cases are not described in the present paper. In postcephalic grafts in reversed anteroposterior and reversed dorsoventral orientation the original polarity apparently always persists. Evidently the orientation and degree of union of the postcephalic graft in relation to the host are factors in determining whether or not the original polarity of the graft persists.

The invagination.—In the ganglionic grafts an invagination from the side of the body opposite the outgrowth is of frequent occurrence, as has been shown above. This invagination may develop from either the dorsal or ventral surface and ranges from a mere depression to a more or less tubular invagination, sometimes extending the whole length of the induced postcephalic outgrowth. In grafts of pieces from postcephalic regions it is also of frequent occurrence, and shows the same range of form; but in certain cases does not persist indefinitely.

This invagination presents another interesting problem. Its development appears to be correlated with the more or less radial character of the outgrowth. The graft determines a localized region of growth in a direction more or less at right angles to the longitudinal body axis. This growth involves not merely the dorsal and ventral surface but the whole thickness of the body, and occurs in all radii in the mediolateral plane of the body in the region where it is localized. The consequence of these conditions is that the outgrowth is more or less cylindrical or conical in form but possesses a dorsoventral differentiation along the radii of its transversed section. Its dorsal surface may be external, its ventral, internal; or vice versa. It appears that such a pattern results simply from the localization of a region of growth involving more or less the whole thickness of the body and at right angles to the polar axis and the mediolateral plane. The outgrowth is more frequently dorsal than ventral; consequently the invagination is more frequently ventral than dorsal. This probably results from the fact that the ventral surface is in contact with the substratum which offers some resistance to the outgrowth.

The controls.—The hosts in which the grafts did not "take" have been used as controls. In most of these, the opening made by the removal of a piece for the insertion of the graft contracts, new tissue develops in it, and the region finally becomes indistinguishable from

adjoining regions. When the piece removed represents the pharyngeal region, a new pharynx develops. When the piece removed is almost the whole width of the body, separation of the parts anterior and posterior to the level of operation often occurs and the parts undergo reconstitution in the usual way. Occasionally the opening left by the removal of the piece does not entirely close, and a head develops in it from the cut surface which faces in the anterior direction.

Transplantation and reconstitution.—In conclusion it may be pointed out that there are certain resemblances between transplantation and the reconstitution of isolated pieces. This is particularly the case with regard to the ganglionic grafts as compared with the regeneration of a head on an isolated piece and its reorganizing effect on levels of the piece posterior to it. Child (1929*b*, pp. 38 and 39 and references there cited) has shown that an isolated piece which remains headless never gives rise to any part anterior to the level of the body from which it was taken, but that, if even a rudimentary head develops, reorganization of the more anterior parts of the piece into those levels of the body which originally lay between the level of the piece and the head takes place. Even in the absence of a head, however, the piece is able to develop all parts representing levels posterior to its level in the original body. This fact indicates that physiological dominance in the reconstitution of *Planaria* acts chiefly in the direction from anterior to posterior. Child has also shown that the scale of organization of the body regions determined by the regenerating head may vary greatly with the degree of development of the head (Child, 1929*b*, pp. 38 and 39 and citations). Evidently the regenerating head is an "organizer" as well as the grafted head or ganglionic region. The chief difference between the results obtained with the ganglionic grafts and the results in reconstitution is that, in the case of the graft, the new head may develop either on the dorsal or the ventral surface of the host and be entirely covered with dorsal or ventral epithelium. Even in such cases it is able to bring about reorganization in the body of the host. In such cases, however, the postcephalic outgrowth may also be completely covered externally with dorsal or ventral epithelium and may contain a tabular invagination lined with the epithelium of the opposite

surface. But even these outgrowths apparently function as postcephalic regions, since further reorganization of more posterior regions in the host body may take place through them. In short, when a head develops at the anterior end of an isolated piece, the effect, as regards reorganization in regions posterior to the regenerating head, is very similar to the effect of a head or ganglionic region grafted at the same body level.

In the case of grafts of postcephalic regions the resemblance to the reconstitution of isolated pieces is not as great. When complete union between graft and host occurs, the graft may or may not give rise to an outgrowth—size of graft, level of body from which it is taken, and level of implantation being factors in determining the result. In other words, these factors determine whether or not the graft is able to grow at the expense of the host and perhaps to bring about some degree of reorganization. Such a graft, however, does not develop a head; and even if it does determine some degree of reorganization, it is much less effective than the ganglionic graft. If, however, the postcephalic graft does not unite completely with the host and develops a head, this head apparently possesses the same ability to reorganize the body of the host as does the head which develops from the ganglionic graft.

In general, the results of the experiments on transplantation agree with the data on reconstitution in showing that the head region is the chief or primary region of physiological dominance in the body.

SUMMARY

1. Grafts of pieces from the ganglionic region of *Planaria dorotocephala* or *P. maculata* implanted in prepharyngeal levels of the host body, if above a certain size, may give rise to heads and determine the development from the host body of a postcephalic outgrowth.

2. Ganglionic grafts implanted into postpharyngeal levels of the hosts not only develop heads, and may determine postcephalic outgrowths from the host body, but also determine a further reorganization in the host body with the development of a pharynx and postpharyngeal region.

3. The reorganizing influence of the graft head may extend in the host body not only posterior but also anterior to the level of the post-

cephalic outgrowth; that is, it may determine two polarities in opposite direction in the host body, one of which represents a complete reversal of the original polarity.

4. The head region which develops from the ganglionic graft is an "organizer" or a "reorganizer"—in other words, a dominant region which initiates and determines the development of postcephalic regions.

5. The scale of organization (that is, the length of the new axis or axes determined by the graft head) varies in different cases.

6. The reorganizing influence of the graft head is not species-specific. Ganglionic grafts from *P. dorotocephala* induce reorganization in *P. maculata*, and vice versa.

7. The head which develops from the ganglionic graft may be normal in structure when union between graft and host is incomplete and dorsal and ventral epithelium of the graft unite with each other. When union is complete, the graft head is a more or less conical or rounded structure, often with a single eye and without cephalic lobes, and is completely covered either with dorsal or ventral epithelium according to the surface on which it develops. Even such heads, however, are able to induce some degree of reorganization in the host.

8. The postcephalic outgrowth induced by the graft head is not a normal postcephalic region. It is more or less cylindrical and is covered either with dorsal or ventral epithelium, according as it develops from the dorsal or ventral surface of the host; and frequently, also, an invagination from the opposite surface of the host body extends a greater or less distance into the outgrowth. This invagination is lined with epithelium characteristic of the surface from which it arises. Consequently the postcephalic outgrowth may be tubular, and possesses dorsal epithelium on its outer and ventral epithelium on its inner surface, or vice versa.

9. The fact that the reorganization of other parts of the host body into pharyngeal and postpharyngeal regions, and even reversal of polarity in the host body, is determined by the graft head through the postcephalic outgrowth indicates that this outgrowth represents physiologically the more anterior postcephalic portion of a polar axis.

10. Various results obtained with grafts of pieces from prepharyngeal and pharyngeal regions are described. When union is complete, such grafts may give rise to headless outgrowths with invagination from the opposite body surface. In cases of incomplete union, reconstitution of a head and reorganization of the graft may occur.

11. In certain cases, the postcephalic grafts migrate from the region of implantation in the host body, and new tissue develops in the region of implantation. With grafts from a given body level implanted in a given level of the host with a given orientation, the direction of migration is apparently always the same. Region of origin and of implantation and orientation of the graft with respect to the host are all factors in determining the direction of migration.

12. In general, when not resorbed, the grafted pieces retain their original dorsoventrality and frequently their original polarity. But in many cases it is impossible to determine whether or not the original polarity persists, and in certain cases in postcephalic grafts it is obliterated and a new polarity determined.

13. The invagination from the opposite side of the body into the outgrowth apparently results from the fact that the graft becomes or induces a localized region of growth in a direction more or less at right angles to the polar axis and the mediolateral plane of the host body and involving the whole thickness of the body. The consequence of such localized growth is the development of an outgrowth from either the dorsal or the ventral surface and a corresponding invagination from the opposite surface.

14. The hosts in which the grafts did not "take" have been used as controls. In most of these the opening made by the removal of a piece for the insertion of the graft contracts; new tissue develops in it; and the region finally becomes indistinguishable from adjoining region. If it is in the pharyngeal region, a new pharynx develops. When the piece removed is almost the whole width of the body, separation of the parts anterior and posterior to the level of operation often occurs and the parts undergo reconstitution in the usual way. Occasionally the opening left by the removal of the piece does not entirely close, and a head develops in it from the cut surface which faces in the anterior direction.

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